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I. THE EXCRETION OF ANTIGEN.

By AGNES ELLEN PORTER.

From the Physiological Department, Edinburgh University, the Lister Institute, London, and the Royal College of Physicians, Edinburgh.

(*Received January 13th, 1915.*)

If egg-white is introduced parenterally into a rabbit, an albuminuria follows. At the same time the animal thus treated becomes immunised. It produces precipitins, which enable its serum to precipitate in the presence of the protein with which it had been originally injected. In this connection the protein is known as antigen. The combination of antigen and antiserum is not only characterised by a precipitate, it possesses also the faculty of binding the complement of fresh normal serum. The withdrawal of complement is tested for by adding sensitised red blood corpuscles, which, in the absence of free complement, do not become haemolysed.

Hamburger [1902] found that the albuminuria which follows injection of egg-white diminished with each re-injection, as the animal became more immune. Oppenheimer [1903] thought the excretion of albumin irregular in amount, bearing no relation to the degree of immunity or to the formation of precipitins. Cramer [1908] investigated this question, and found that the albuminuria did diminish with further injections of egg-white, but that the amount of albumin excreted depended still more on the stage of digestion. A fasting animal did not make use of the albumin injected into the peritoneal cavity as well as a digesting animal, unless it had received a previous injection of saline solution to induce a local leucocytosis. If the leucocytes took up the protein it was digested by the leucocytic proteolytic ferments and assimilated. Failing a local leucocytosis, it was not assimilated to the same extent and was excreted in much greater amount through the kidney cells. The amount excreted was always less than the amount injected. Cramer also examined chemically the albumin in the urine, and found that it gave every

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characteristic chemical test of albumin and that in its behaviour to alcohol and ether it resembled egg-albumin and not serum-albumin. Yet it differed from the original egg-albumin in that it was incapable of forming a precipitate with anti-egg serum. Moreover, it did not give a precipitate with anti-rabbit serum. It was apparently egg-albumin which had lost its "species specificity."

There are two ways in which egg protein may be tested for specificity, first, in vitro, by precipitating with anti-egg serum, secondly, in vivo, by producing precipitin in the serum of the animal into which it has been injected. At Dr Cramer's suggestion, I took up this question in order to determine whether this excreted albumin was still antigen in the sense of being capable of producing antibody when injected into another rabbit, also whether, when mixed with anti-egg serum, it could bind complement.

The Excreted Albumin Tested as Antigen in Vivo.

The albumin was salted out of the urine by saturation with ammonium sulphate, was redissolved and reprecipitated, and was finally dialysed in parchment sacs to remove the precipitating agent. In spite of this treatment it retained a considerable amount of urinary pigment. The urine used for this purpose was that from Rabbit 1 of Group 1, taken on the first and second days after each of four successive injections of egg-white; its behaviour as antigen in vitro will be discussed later. After dialysis, this excreted albumin was thrice injected intraperitoneally into one rabbit, at five-day intervals. On the third occasion the injection was followed within a few minutes by convulsions and death, with all the appearance of anaphylaxis. Blood was taken from the heart immediately after death, and the serum separated from it contained no precipitin. A control rabbit, injected with egg-white which had been similarly precipitated with ammonium sulphate, and then dialysed, did produce precipitin, but of a very low titre, 1/100.

The Excreted Albumin Tested as Antigen in Vitro.

An investigation into the influence of excreted albumin upon anti-egg serum in vitro disclosed, in the first group of rabbits examined, a curious phenomenon.

Three groups of rabbits were used, each group in a different year, season of the year, and laboratory, under different feeding conditions; different

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samples of serum were also used in the three groups of experiments. It is interesting that each group of rabbits excreted an albumin which differed from the albumin excreted in the other two groups in its properties as antigen.

Group 1 comprised three rabbits, four times injected, yielding twelve samples of excreted albumin. These samples all possessed the same properties as antigen. They failed completely to precipitate with anti-egg serum. When mixed with anti-egg serum, they exhibited the curious phenomenon of completely inhibiting it from precipitating with fresh egg-white. Although the mixture of excreted albumin and anti-egg serum did not precipitate, it bound complement.

Group 2 consisted of three rabbits, four times injected, yielding twelve samples of excreted albumin. These all failed to precipitate with anti-egg serum, but only one of them exerted any inhibitory influence at all (and that only partial) on precipitation between anti-egg serum and fresh egg-white. They also were unable to bind complement.

Group 3 was represented only by one rabbit, once injected. This sample of excreted albumin precipitated with anti-egg serum, and, with it, bound complement.

The animals were placed in cages, and their urine tested as to quantity, reaction, percentage of urea, and freedom from albumin. They then received an intraperitoneal injection of 10 to 13 cc. egg-white. The urine of the first and second days after injection was examined similarly for quantity, reaction, urea, and albumin. The urine seldom contained more than a trace of albumin after the second day after each injection. The rabbits of the first group were investigated in very cold weather. They were small, about 1·5 kilos. in weight, were poorly fed on bran and water, and showed suppression of urine for 12 to 24 hours after injection. Those of the second group were investigated in hot weather, they were large rabbits above 2·5 kilos. in weight, and were well fed on corn and carrots. No consistent alteration could be observed in the reaction or quantity of urine passed during the day after injection, though there was a rise in the percentage of urea constantly present. The single rabbit representing Group 3 was investigated in spring, it weighed 2 kilos., was fed on corn and turnips, and the urine was increased in total quantity on the day after injection. On the day before injection it was as low as 120 cc., the day after injection 225 cc., the second day after, it rose to 375 cc.

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Table from Group 1 showing Inhibition.

(By "immune" urine is meant the albuminous urine of a rabbit which had been injected with egg-white.)

					Precipitate
1.	0·2 cc. Anti-egg serum	+	0·2 cc.	Normal urine 1	0
2.	,, ,,	+	,,	Immune urine 1	0
3.	,, ,,	+	,,	0·85 % NaCl	0
4.	,, ,,	+	,,	1/10 Egg-white in NaCl	++
5.	,, ,,	+	,,	,, ,, normal urine	++
6.	,, ,,	+	,,	,, ,, immune ,,	+
7.	,, ,,	+	,,	NaCl + 0·2 cc. 1/10 Egg-white	++
8.	,, ,,	+	,,	Normal urine + ,, ,, ,,	++
9.	,, ,,	+	,,	Immune ,, + ,, ,, ,,	0
10.	,, ,,	+	,,	NaCl + ,, 1/100 ,,	++
11.	,, ,,	+	,,	Normal urine + ,, ,, ,,	++
12.	,, ,,	+	,,	Immune ,, + ,, ,, ,,	0

In order to obtain this inhibition it was necessary to add the urine first to the antiserum, before the fresh egg-white (see Exp. 6). If excreted albumin and fresh egg-white were added together, as where egg-white was dissolved 1/10 in the immune (already albuminous) urine (Exp. 9), the inhibition was only partial.

Complement Binding Experiment.

(Two drops of fresh guinea pig serum were added from a capillary pipette to a series of tubes containing antiserum, and NaCl, urine, or egg-white. After one hour at room temperature 0·5 cc. red corpuscles 2 % were added with immune body. The tubes were then placed at 37° for one hour.)

					Haemolysis
1.	0·2 cc. Antiserum	+	0·2 cc.	Normal urine	complete
2.	,, ,,	+	,,	Immune ,,	trace
3.	,, ,,	+	,,	0·85 % NaCl	complete
4.	,, ,,	+	,,	Normal urine + 0·2 cc. 1/10 Egg-white	0
5.	,, ,,	+	,,	Immune ,, + ,, ,,	0
6.	,, ,,	+	,,	NaCl + ,, ,,	0
7.	0·4 cc. NaCl			+ ,, ,,	complete

Table from Group 2 showing Indifference.

Urine "Rabbit 2" of Group 2 exhibited after the first injection a partial inhibition which was not observed after later re-injections. As it was atypical of the group, the results obtained with urine from Rabbit 1 of the group will be represented.

					Precipitate
1.	0·2 cc. Anti-egg serum	+	0·2 cc.	Normal urine 1	0
2.	,, ,,	+	,,	Immune urine 1	0
3.	,, ,,	+	,,	0·85 % NaCl	0
4.	,, ,,	+	,,	Normal urine + 0·2 cc. 1/10 Egg-white	++
5.	,, ,,	+	,,	Immune ,, + ,, ,, ,,	++
6.	,, ,,	+	,,	NaCl + ,, ,, ,,	++
7.	,, ,,	+	,,	Normal urine + ,, 1/100 ,,	++
8.	,, ,,	+	,,	Immune ,, + ,, ,, ,,	++
9.	,, ,,	+	,,	NaCl + ,, ,, ,,	++

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Complement Binding Experiment.

(Method as above.)

		Haemolysis
1.	0·2 cc. Antiserum + 0·2 cc. Normal urine	complete
2.	„ „ + „ Immune „	„
3.	„ „ + „ 0·85 % NaCl	„
4.	„ „ + „ Normal urine + 0·2 cc. 1/10 Egg-white	0
5.	„ „ + „ Immune „ + „ „	0
6.	„ „ + „ 0·85 % NaCl + „ „	0

Table from Group 3 showing Unimpaired Antigen.

		Precipitate
1.	0·2 cc. Antiserum + 0·2 cc. Normal urine	0
2.	„ „ + „ Immune „	++
3.	„ „ + „ 0·85 % NaCl	0
4.	„ „ + „ Normal urine + 0·2 cc. 1/10 Egg-white	++
5.	„ „ + „ Immune „ + „ „	++
6.	„ „ + „ 0·85 % NaCl + „ „	++
7.	„ „ + „ Normal urine + „ 1/100 „	++
8.	„ „ + „ Immune „ + „ „	++
9.	„ „ + „ 0·85 % NaCl + „ „	++

Complement Binding Experiment.

(Method as above.)

		Haemolysis
1.	0·2 cc. Antiserum + 0·2 cc. Normal urine	complete
2.	„ „ + „ Immune „	0
3.	„ „ + „ 0·85 % NaCl	complete
4.	„ „ + „ Normal urine + 0·2 cc. 1/10 Egg-white	0
5.	„ „ + „ Immune „ + „ „	0
6.	„ „ + „ 0·85 % NaCl + „ „	0

These experiments indicate that albumin may be excreted by the kidneys, after intraperitoneal injection of egg-white, in three conditions, as regards the precipitin reaction. In Group 3 its antigen properties were unimpaired in every respect. In Group 1 while chemical properties were retained, the power of precipitating was lost; in view of the inhibition, the albumin may be described as having retained its haptophore affinities. In Group 2 chemical reactions being again unchanged, both precipitating and haptophore properties were lost. It is no easy matter to explain the occurrence of these three conditions of albumin in urine. Michaelis [1903] states that an excess of antigen inhibits the precipitation, though Chapman [1910] finds that excess of antigen does not affect the reaction in either direction. In any case this explanation does not meet the present case. The inhibition was sometimes exerted by urine containing only a trace of albumin, and not by

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urine containing much. Also, as has been already stated, fresh egg-white, dissolved in albuminous and inhibitive urine, while increasing the total protein present, diminished the inhibition, which was always greater where the inhibiting urine was added first to the antiserum than where both forms of protein were added together.

Another possible explanation of the loss of precipitating power, and of acquisition of inhibitory influence might lie in the action of the urea. Limbourg [1887] found that urea has a very powerful solvent action upon protein. Spiro [1900] observed that fibrin and coagulated egg-white were dissolved by urea at 37°, and could not be recoagulated by the application of heat as long as the urea was present. If however the solution of fibrin in urea was dialysed and the urea thereby removed the coagulability of the protein returned. Pick [1902] found that saturated urea solution (50 % in water) prevents the precipitin reaction through its action upon the serum protein. This fact I am able to confirm in the concentration, 50 %, which Pick used. I could find, on the other hand, no influence whatever in the case of 5 % urea. Egg-white dissolved in 0·85 % salt solution containing 5 % urea, did not lose as antigen in the slightest degree. If 5 % urea in salt solution was added to anti-egg serum in equal proportions no inhibition to the action of egg-white could be detected, other than by diluting the serum by one-half, an action in which it was equalled by control salt solution. It may be stated that although the percentage of urea in the urine rose after injection, it on no occasion rose as high as 0·5 %. It is possible that albumin may be subjected to a high percentage of urea in the kidney tubules, especially where suppression of urine occurs. Egg-white was therefore treated with an equal quantity of saturated urea solution at 0°, at room temperature, at 37° for 24 hours, and at 56° for two hours. The egg-white was then diluted to 1/10 in salt solution, making 1/10 egg-white in 5 % urea solution. No impairment of antigen properties could be detected. Egg-white diluted 1/10 in saturated urea solution, and after 24 hours treatment diluted to 1/100 in salt solution, did not appear to differ in any way from the control experiment with 1/100 urea solution. This result agrees with Spiro's observation that the solvent action of urea does not consist in any permanent change in the protein; if the urea is removed either by dialysis or dilution to an innocuous concentration, the solvent action disappears. This makes it clear that the action of urea in the kidney tubules is not responsible for the change in the albumin excreted in the urine. The concentration of the urea passed, under 0·5 %, was too low to inhibit precipitation.

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The urine normally contains pepsin and trypsin [Sahli, 1885]. Pepsin and trypsin act for some time on protein changing its physical condition before a point is reached at which chemical protein reactions are changed or lost. Klug [1902] has found for pepsin, and Roberts [Oppenheimer, 1909] for trypsin, that increase of temperature, at least between such limits as 0° and 40°, has an accelerating influence upon protein digestion. Now the albumin of Group 2, where the antigen was most affected, was excreted in very hot weather. On the other hand, the weather was warmer in the third case (in spring), where the albumin was unimpaired, than in the first, where the albumin had lost its precipitating power, and where there was much cold and frost. It must be mentioned that toluene and thymol were placed in the urine bottles.

The first step in protein digestion is the most difficult. This means that protein digestion is unlikely to take place in "layers"; that is to say, when a part of the protein present has reached a certain stage, the succeeding stages are rapid, so that all stages of protein digestion may be present coincidently.

I treated egg-white with 0·3 % trypsin in N/100 NaOH, and found that this enzyme had little or no action in 24 hours even in the presence of urea on antigen properties. Pepsin had a far greater action. I used 1/500 Ray's pepsin in N/50 acid, testing the treated egg-white in 0·5 hour, 1 hour, 1·5 hours, etc., after treatment at 37°, and room temperature. The antigen properties were quickly weakened, but a trace of precipitating power was left until the chemical reactions for albumin had also disappeared. When the egg-white was shown by chemical tests to be largely digested, the precipitating power was almost gone.

From these results it appears improbable that the deterioration of specific egg-white can be ascribed either to the action of the urea, or of pepsin and trypsin present.

Haswell Wilson [1909] has found that albumin excreted in human urine in disease is constant in its action as antigen in the complement binding test, but he was dealing with diseased kidneys, whereas in the rabbits under investigation the kidneys were normal. I am unable to find an explanation of the change of specificity in this albumin, other than that of a physical nature, such as Cramer has indicated. Cramer points out that these results are incompatible with the generally accepted view that the power of protein to act as antigen is dependent on a specific chemical group in the protein molecule. These facts, according to him, make it necessary to abandon this view and to assume that the property of protein to act as antigen either

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depends on the physical state of the protein molecule, or that it is due to a substance which is always associated with protein in protoplasm without forming part of the protein molecule, and which can be separated from it without altering the purely chemical properties of the protein.

This research was partly carried out with the help of a Lister and a Carnegie Research Scholarship.

Conclusions.

Egg-white introduced parenterally into a rabbit is excreted unchanged in chemical properties, but may be in three possible variations as regards specificity:

1. It may retain partial properties as antigen, binding complement in the presence of anti-egg serum, not causing precipitation but capable of exercising an influence over antibody so as to prevent it precipitating with fresh antigen.

2. It may be excreted in a non-specific form completely indifferent as antigen.

3. It may retain full antigen properties.

This variation in specificity of excreted albumin points to a physical rather than a chemical explanation of the precipitin reaction.

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II. THE TOXICITY OF SODIUM PYROPHOSPHATE ADMINISTERED IN FOOD; WITH A NOTE ON TOXIC COTTON SEED MEAL.

By WILLIAM LEGGE SYMES and JOHN ADDYMAN GARDNER.

From the Physiological Laboratory of the University of London, South Kensington.

(*Received January 23rd, 1915.*)

Our attention was drawn to the comparative physiological activity of ortho- and pyrophosphate of sodium in the course of observations on the physiological effects of some camphene-phosphonates, which appeared to bear to one another the same relation as that which exists between the phosphates in question.

In the course of this work [1911] we compared the activity of the phosphates, in solution, by intravenous injection, and confirmed the findings of earlier workers, viz., that the orthophosphate is relatively inert, whilst the pyrophosphate is much more toxic.

We attributed the activity of the pyrophosphate, in part, to the alkalinity of its solutions, in part to its acid radicle. Its solutions after "neutralisation," with hydrochloric acid, to litmus, to phenolphthalein, and to methyl orange, though less active, were still markedly toxic. Almost simultaneously with the above, Starkenstein [1911] referred the toxicity of pyrophosphate solutions, in part to their anions, in part to their kations. He further stated, in agreement with Gamgee, Priestley, and Larmuth [1877], that pyrophosphate administered by the mouth was not poisonous.

Crawford [1910] on the other hand, administered sodium pyrophosphate to rabbits, by the mouth, with fatal results. The salt, given in doses of 0·5–1·0 g. dissolved in 25–40 cc. of water, was usually lethal within three days, the post-mortem appearances described by him, suggesting that the deaths were due to an irritant poison.

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It seemed therefore worth while to test afresh the results of administering the salt by the alimentary canal. This has been done on rabbits, cats, and rats, with the result that large doses, administered for prolonged periods, have failed to produce acute poisoning.

A sheep, on the other hand, died after taking 21 grams of the salt.

In this, as in our earlier paper, the chemical side has been the work of Gardner, whilst the physiological portion is that of Symes.

Observations on Rabbits.

The first set of observations was made on three pairs of young rabbits, fed with the same food, viz., oats, bran, and chopped cabbage.

The first and second pairs received daily doses, of ortho- and of pyrophosphate of sodium respectively, whilst the third was kept to control the effect of outside factors. In the first two cases (pairs) the salt was powdered, mixed with 15 g. of chopped cabbage, and given to each animal separately. This ensured complete ingestion of the salt by each individual, as the animals devoured the cabbage with relish. Apart from the above the animals were fed, and housed, in pairs. They were weighed, singly, at the commencement of the experiment, and at intervals of seven days during its continuance. Table I shows the result of four weeks of this régime. The doses were doubled each week and, molecule for molecule, that of the orthophosphate was much the greater.

TABLE I.

Body-weights (weekly) of young rabbits, fed on a mixed diet, with and without addition of phosphate.

	On Na_2HPO_4 12 Aq.		Daily dose per head	On $Na_4P_2O_7$ 10 Aq.		Daily dose per head	No added phosphate	
Week	Rabbit A	Rabbit B		Rabbit C	Rabbit D		Rabbit E	Rabbit F
	Kg.	Kg.	g.	Kg.	Kg.	g.	Kg.	Kg.
0	0·90	0·90	—	0·80	0·60	—	1·10	0·60
1	1·10	1·00	0·32	0·98	0·80	·13	1·40	0·82
2	1·10	1·05	0·64	1·00	0·77	·26	1·40	0·80
3	1·15	1·10	1·3	1·10	0·70	·52	1·45	0·90
4	1·20	1·10	2·6	1·00	0·80	1·0	1·70	1·00
Increase in body-weight	33 %	22 %		25 %	33 %		55 %	67 %

The above figures suggest that, although neither phosphate proved acutely poisonous, each one had, at this stage of life, an adverse influence on growth. This influence was not maintained in later observations on the same animals.

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On completion of these observations the animals were all kept on the same mixed diet, without phosphate, for eight weeks. During this period their weights increased as follows:

Rabbit	A	from	1·20 kg.	to	1·75 kg.	= 46 %
	B		1·10		1·35	= 23
	C		1·00		1·60	= 60
	D		0·80		0·95	= 19
	E		1·70		1·80	= 6
	F		1·00		1·35	= 35

The growth of rabbit D during this period is, for its weight, strikingly small. This animal was, from the commencement, weakly, and light in proportion to its apparent bulk, and its subsequent death did not appear to be primarily due to the experiment.

After this interval (eight weeks), the animals were again treated as shown in Table I, with the results given in Table II.

TABLE II.

Weekly body-weights of rabbits as in Table I.

	On Na_2HPO_4 12 Aq.		Daily dose	On $Na_4P_2O_7$ 10 Aq.		Daily dose	No added phosphate	
Week	Rabbit A	Rabbit B	per head	Rabbit C	Rabbit D	per head	Rabbit E	Rabbit F
	Kg.	Kg.	g.	Kg.	Kg.	g.	Kg.	Kg.
0	1·75	1·35	—	1·60	0·95	—	1·80	1·35
1	1·70	1·70	0·35	1·60	0·80	0·23	1·80	1·30
2	1·80	1·50	0·71	1·80	1·00	0·46	1·90	1·35
3	2·10	1·70	1·4	1·90	1·30	0·93	1·85	1·50
4	2·30	1·70	2·8	1·90	1·45	1·9	2·00	1·70
5	2·30	1·85	„	2·00	1·50	„	2·20	1·80
6	2·40	2·00	„	2·00	1·60	„	2·15	1·90
7	2·45	1·90	„	2·15	1·50	„	2·20	1·95
8	2·50	1·90	„	2·30	1·60	„	2·10	2·00
9	2·70	2·30	„	2·20	1·50	„	2·15	2·00
10	2·65	2·20	„	2·20	1·20	„	2·25	2·20
11	2·60	2·10	1·8	2·15	1·10	1·2	2·30	2·25
12	2·70	2·20	„	2·20	1·10	„	2·25	2·20
Increase in body-wt.	54 %	63 %		38 %	16 %		25 %	63 %

The doses were doubled each week for the first month. That of the orthophosphate was, again, the greater.

Apart from Rabbit D, these figures do not suggest that either phosphate had, with the older rabbits, any adverse effect on growth. This animal which, as above stated, had appeared weakly from the beginning, died about a week after the observations were completed.

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The autopsy revealed no gastric or intestinal lesion, nor anything to show that the death resulted from the pyrophosphate.

Towards the end of this series one rabbit of each group was isolated, for one week, for collection of its urine and faeces.

In each case the salt was eaten in full. On estimating the P_2O_5 in the dejecta, by Neumann's method, the whole of the P_2O_5 given, as orthophosphate, was recovered; whilst the P_2O_5 recovered from the urine and faeces of the rabbit taking 2·3 g. P_2O_5 as pyrophosphate only slightly exceeded that from the control animal.

As already mentioned, the whole of the pyrophosphate was eaten. The missing P_2O_5 was not retained in the animal, but deposited as "earthy phosphate" on the floor of the cage, from which it was not dislodged by the water with which the cage was sluiced at the end of each week, but was subsequently, in great part, recovered by washing with hydrochloric acid.

Two further experiments yielded the same result, i.e. all the P_2O_5 given as Na_2HPO_4 was recovered from the dejecta plus water washings; whilst much of that given in $Na_4P_2O_7$ was found as an almost invisible deposit on the floor of the cage.

On this account, the experiment was repeated on a cat, in the anticipation that the high acidity of its urine would diminish or prevent the deposition of the phosphates.

Observations on Cats.

The results on two cats were as follows.

The first, a full grown but rather thin cat, was given (and ate) 2 g. of $Na_4P_2O_7$, 10 aq., daily, for 15 days, mixed with soaked biscuit and milk. The animal showed no sign of discomfort and gained 100 g. in weight during this period, in spite of slight diarrhoea.

The second, a younger cat, was given (and ate) the same dose for 13 days. This animal vomited once, but gained 100 g. in weight during the observation. The same cat, two months later, took 2 g. of Na_2HPO_4, 12 aq., daily, for 9 days, also without any symptom of acute poisoning. Both phosphates produced slight diuresis, and some tendency to diarrhoea. In consequence of the latter, the faeces were not collected. The urine, collected during a week of each régime, in comparison with that of a week on the same diet without addition of phosphate yielded P_2O_5 as shown in the following table.

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TABLE III.

Urine of a cat on a mixed diet, and on the same diet plus phosphate.

Régime	Volume (one week)	Sp. Gr.	P_2O_5 recovered	P_2O_5 given as salt	Difference between P_2O_5 recovered, and P_2O_5 given as salt
Control	748 cc.	1026	2·0 g.	0·0 g.	2·0 g.
Orthophosphate	819 cc.	1040	4·7 g.	2·4 g.	2·3 g.
Pyrophosphate	776 cc.	1038	5·5 g.	3·8 g.	1·7 g.

It is obvious that both phosphates were absorbed, and that loss by deposit was not great.

Observations on Rats.

A series of observations was made on three pairs of rats. The animals were fed on bread moistened with water and on oats. The first pair was given, daily, with the soaked bread, 0·5 g. of Na_2HPO_4, 12 aq.; the second pair an equal weight of $Na_4P_2O_7$, 10 aq.; whilst the third pair had no phosphate added to its ration. The animals were fed in pairs, and weighed (also in pairs) once a week. Table IV shows the body-weights under nine weeks of this régime.

TABLE IV.

Body-weights (weekly) of pairs of white rats, fed on a mixed diet, with and without addition of phosphate.

Week	On Na_2HPO_4 12 Aq. 0·5 g. daily	On $Na_4P_2O_7$ 10 Aq. 0·5 g. daily	No added phosphate
0	217 g.	175 g.	229 g.
1	229 ,,	193 ,,	233 ,,
2	232 ,,	197 ,,	248 ,,
3	235 ,,	192 ,,	254 ,,
4	238 ,,	206 ,,	267 ,,
5	244 ,,	213 ,,	263 ,,
6	259 ,,	224 ,,	263 ,,
7	256 ,,	220 ,,	273 ,,
8	256 ,,	222 ,,	277 ,,
9	245 ,,	219 ,,	285 ,,
Increase in body-weight	13 %	25 %	25 %

The pyrophosphate pair, starting 25 % below the controls, grew at the same nett rate as these, whilst the orthophosphate pair starting at the same level (as the controls) grew less rapidly. Both of the phosphate pairs lost weight after the sixth week.

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It was noted that the pyrophosphate ration was eaten with less relish than the others, and that on 21 days (out of the 63) some of it was left, thereby entailing loss of some of the phosphate. But since on the remaining 42 days the dose was fully ingested, the table confirms the indications of the earlier series, that neither phosphate was acutely poisonous.

Observations on a Sheep.

On account of the supposed presence, and toxicity, of pyrophosphate in some samples of cotton seed meal, it was thought well to test the effect of pyrophosphate on a sheep, and we are indebted to the Director of the Lister Institute for affording us facilities for this portion of the work.

A full grown wether was given, daily, 1·0 g. of $Na_4P_2O_7$, 10 aq., in 50 g. of dry oatmeal, in addition to hay, for a week. The meal was eaten with relish and produced no noticeable effect. After a week's interval, the daily dose of pyrophosphate was recommenced and doubled. This was still taken with relish, but produced some thirst and restlessness, and the animal was found dead on the morning following the seventh dose, i.e. after a total intake of 21 g. of pyrophosphate.

The autopsy showed marked softening of the mucous membrane of the rumen and a small patch of inflammation, in its wall, opposite the spleen, to which it was firmly adherent.

The reticulum and omasum appeared normal, whilst the abomasum and small intestine were inflamed throughout. No ulcer was seen in any portion of stomach or intestine. This result agrees with those of Crawford, on rabbits, in which the conditions of experiment were substantially those in the case of the sheep. Crawford administered $Na_4P_2O_7$ in (approximately 2 %) solution, i.e. he introduced a strongly alkaline solution into the stomachs of his animals.

Introduction of dry $Na_4P_2O_7$ into the already alkaline contents of the sheep's rumen yields, likewise, a strongly alkaline solution. This alkaline solution produced the same effect as Crawford's more concentrated alkaline solutions, though without visible ulceration and less rapidly.

To our rabbits, the pyrophosphate was given as powder in chopped cabbage; to the rats and cats, as powder in moistened bread and biscuit respectively. In these cases, the salt was presumably, in great part, (if not wholly) neutralised as rapidly as it reached the stomach wall. It certainly produced no acute poisoning.

Equally certainly it was largely absorbed, as shown by the analyses of

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the urines, and urinary deposits. It would appear, therefore, either that the pyrophosphoric acid, liberated by the gastric juice, was hydrolysed to the less active orthophosphoric acid (as suggested by Starkenstein), or that (as supposed by Gamgee) the absorption of pyrophosphate was balanced by its prompt excretion.

Note on Toxic Cotton Seed Meal.

In relation to cotton seed poisoning, mentioned above, we may add that a sample of toxic cotton seed meal which has come into our hands, has proved fatal to rats. Four of these animals, fed exclusively (and *ad libitum*) on the meal, died on the 5th, 7th, 13th and 14th days respectively, showing gastro-intestinal inflammation without visible ulceration or noticeable softening of the mucous membrane. The appearances were, in short, those common to irritant poisons, not distinctive of any particular one.

As detailed above, our rats consumed, daily, 0·5 g. per head of crystalline sodium pyrophosphate (= 0·3 g. $Na_4P_2O_7$), for nine weeks, without visible harm. On the other hand, four rats fed on oats, and bread, plus 5 g. of cotton seed meal per head, died on the 10th, 15th, 24th, and 25th days respectively. Four other rats fed on oats, bran, and 5 g. per head of the same cotton seed meal, after previous extraction with alcohol and with ether, lived 26, 31, 40, and 50 days respectively; whilst four (control) rats, fed on oats and bread only, maintained their health and gained 77 g. in weight, in the 50 days of the experiment. The P_2O_5 content of the (air dry) cotton seed meal was 0·43 %, and amounted to 0·022 g. in the daily ration. This corresponds to 0·07 g. of $Na_4P_2O_7$, 10 aq., i.e. less than one-seventh of the quantity tolerated, daily, for nine weeks. It is clear from the reduction of toxicity by alcohol and ether, and from the low phosphate content of the seeds that sodium pyrophosphate cannot be the chief poison in the meal in question.

It was hoped to identify the poisonous principles and to extend the observations to ruminants, but the non-renewal of a small grant from the Board of Agriculture has rendered this, for the present, impracticable.

Summary and Conclusions.

Sodium pyrophosphate administered, with food, to rabbits, cats, and rats, is devoid of the toxic action that it shows when intravenously injected. This confirms the verdict of Gamgee and his pupils, and of Starkenstein.

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Administered to a sheep, in the same way, it has proved lethal, producing effects similar to those described, by Crawford, as following its administration, in aqueous solution, to rabbits.

Such toxic action as sodium pyrophosphate exerts, when administered by the mouth, differs from that of the same compound, intravenously injected, in that it is wholly due to the alkalinity of the salt and not to the acid radicle.

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III. NOUVELLES RECHERCHES SUR LA DIGESTION DES PROTÉINES DE LA VIANDE CUITE CHEZ LE CHIEN (SECONDE COMMUNICATION).

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I. INTRODUCTION.

Dans un travail antérieur [1913], j'ai utilisé la centrifugation pour séparer, chez le chien, les protéines et leurs diverses espèces de dérivés dans le contenu tant de chacune des deux portions principales de l'estomac que de la première portion de l'intestin grêle à divers moments après l'ingestion de viande cuite de cheval. J'ai ainsi comparé la répartition de l'azote entre les protéines intactes, l'acidalbumine, les protéoses et les autres dérivés des substances albuminoïdes, selon qu'on emploie pour cette séparation l'ancienne méthode par filtration ou le nouveau procédé par centrifugation. Ce dernier permet d'éviter les modifications dans la composition des filtrats, signalées par *Christiansen* [1912].

Après l'ingestion de viande de cheval, on trouve en moyenne une proportion un peu plus considérable d'acidalbumine et de protéoses considérées ensemble, et par conséquent une proportion un peu moindre des autres produits de désintégration des protéines dans les diverses régions du tube digestif lorsqu'on emploie la centrifugation que lorsqu'on utilise la filtration. Il semble que pendant le laps de temps assez long exigé parfois par les filtrations successives nécessitées par la détermination de la répartition de l'azote entre les protéines et leurs diverses espèces de dérivés, une partie, relativement faible il est vrai, de l'acidalbumine et des protéoses se transforme en peptones et en substances abiurétiques. Il se peut aussi que les filtres retiennent par adsorption plus facilement l'acidalbumine et les protéoses que les peptones et les dérivés abiurétiques des protéines.

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Quoi qu'il en soit, on doit renoncer à la méthode par filtration et lui préférer celle, plus rapide et plus exacte, par centrifugation. A coup sûr, bien que les différences entre les résultats obtenus par les deux procédés ne soient pas très considérables pour ce qui concerne, chez le chien, la digestion de la viande cuite de cheval, elles méritent néanmoins d'appeler l'attention. Aussi m'a t'il paru utile d'appliquer la méthode par centrifugation à l'étude de la digestion par le chien de la viande cuite de bœuf, afin de voir si les différences signalées ci-dessus entre les résultats des deux procédés à propos de la viande cuite de cheval se vérifiaient pour celle de bœuf.

J'ai, en outre, profité de ces expériences pour rechercher la teneur en azote ammoniacal et en azote aminé des diverses régions du tube digestif au cours de la digestion de la viande cuite de bœuf.

II. Technique.

On a débarrassé les chiens au préalable des vers intestinaux [Zunz, 1910, 1]. On a maintenu ces animaux 24 à 36 heures à jeûn, tout en leur permettant de boire de l'eau à volonté. On leur a ensuite donné 25 grammes de viande cuite de bœuf par kilogramme de poids. On a déterminé, par la méthode de *Kjeldahl*, la teneur en azote de chaque échantillon de viande après dissolution dans l'acide sulfurique.

On a sacrifié les 12 chiens A à L, 1 à 12 heures après le repas, par piqûre du bulbe et saignée des carotides. On a rapidement incisé la paroi abdominale en ayant soin de ne pas bouger aux viscères. On a isolé, par le procédé que j'ai décrit [1910, 1], les deux régions principales de l'estomac et la première portion de l'intestin grêle.

Dans mes communications antérieures j'ai conservé aux deux parties essentielles de l'estomac du chien les dénominations de fundus et de région ou d'antre prépylorique, adoptées par beaucoup de physiologistes. Mais si l'on considère les travaux les plus récents des anatomistes et des radiologues, il vaut mieux appeler corps de l'estomac la portion de cet organe désignée jusqu'à présent sous le nom de fundus; elle comprend le corpus gastricus, le canalis gastricus et le fundus proprement dit ou sac cardiaque. Quant à la portion prépylorique, elle correspond à la fois à l'antrum pyloricum et au vestibulum pyloricum des anatomistes.

On a recueilli séparément dans de l'eau distillée le contenu du corps de l'estomac, celui de la portion prépylorique de cet organe et celui de la première partie de l'intestin grêle. On a aussitôt soumis ces bouillies alimentaires

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à l'ébullition, après avoir ajouté le cas échéant de l'acide chlorhydrique dilué au contenu intestinal afin de le rendre légèrement acide, puis on les a laissé refroidir.

On a séparé, par une centrifugation de 10 à 15 minutes, à la centrifugeuse Jouan, tournant à la vitesse de 7000 tours par minute, les matières non attaquées et les protéines coagulées du liquide contenant l'azote soluble. On a remis le précipité en suspension dans de l'eau distillée et l'on a procédé à une nouvelle centrifugation. On a répété ces deux opérations quatre à six fois, c'est-à-dire jusqu'au moment où le liquide surnageant n'a plus donné la réaction du biuret.

Tant pour le contenu du corps de l'estomac que pour celui de la portion prépylorique de cet organe, on a traité par l'acide sulfurique la partie non dissoute du bol alimentaire, et l'on a dosé, par la méthode de *Kjeldahl*, l'azote coagulable de ces solutions.

On a réduit, le cas échéant dans le vide, à une température ne dépassant pas 35 à 40° C., les liquides surnageants et les eaux de lavage renfermant l'azote soluble non coagulable, de manière à obtenir, pour le contenu de chacune des trois portions du tube digestif examinées, un filtrat initial, dont le volume total atteigne 150 à 200 cm.^{3} On a prélevé de chaque filtrat initial: 1° trois portions de 5 cm.^{3} destinées au dosage de l'azote soluble non coagulable par la méthode de *Kjeldahl*; 2° deux portions de 20 cm.^{3} destinées au dosage de l'ammoniaque par la méthode de *Folin*; 3° deux portions de 10 cm.^{3} destinées au dosage de l'azote aminé aliphatique par la méthode de *van Slyke*.

On a effectué cette dernière détermination en suivant exactement les indications les plus récentes du savant américain [van Slyke, 1912, 1913]. On s'est servi, dans ce but, du nouvel appareil imaginé par cet auteur afin de permettre une agitation continue de même intensité pendant les cinq minutes d'action de l'acide nitreux, de telle sorte que les conditions de la réaction restent identiques dans tous les cas. J'ai toutefois fait modifier légèrement cet appareil, de façon à augmenter la précision des lectures. La burette renfermant le liquide à analyser est divisée en cinquantièmes de centimètre cube. Les six premiers centimètres cubes de la burette gazométrique sont gradués en vingtièmes de centimètre cube. On a calculé en milligrammes l'azote ammoniacal et l'azote décelable par la méthode de *van Slyke* renfermés dans l'ensemble du filtrat initial. On a soustrait du chiffre ainsi calculé par la méthode de *van Slyke* le tiers de l'azote ammoniacal. Le tableau I indique les chiffres d'azote aminé ainsi corrigés.

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J'ai exposé antérieurement les raisons pour lesquelles j'avais adopté cette façon de procéder dans les recherches sur la digestion des protéines de la viande cuite de cheval. Afin de pouvoir comparer les résultats obtenus de cette manière et ceux des nouvelles recherches sur la digestion de la viande cuite de bœuf, j'ai eu soin d'employer la même technique dans ces deux séries d'expériences. La technique plus perfectionnée, basée sur le procédé de désalbuminisation de *van Slyke* et *Meyer* [1912] et sur l'emploi d'uréase de la façon indiquée par *Marshall* [1913], n'est, du reste, pas très aisée à appliquer lorsqu'on doit opérer avec un liquide ne renfermant que très peu d'azote aminé.

Le restant du filtrat renfermant l'azote soluble non coagulable sert à déterminer, par la méthode que j'ai proposée [1910,1], successivement l'azote de l'acidalbumine, l'azote de l'hétéroalbumose et de la protoalbumose[1], l'azote des autres protéoses, l'azote des dérivés des protéines précipités par l'acide phosphotungstique mais pas par le sulfate de zinc, l'azote des composés précipités ni par l'un ni par l'autre de ces réactifs en ayant soin de séparer les précipités successifs par une centrifugation énergique de 10 à 15 minutes, puis de décanter les liquides parfaitement clairs ainsi obtenus.

On a aussi examiné si les liquides débarrassés des protéines, de l'acidalbumine et des protéoses, donnaient encore la réaction du biuret décelant la présence des peptones vraies de *Kühne*.

III. Résultats.

Dix tableaux rassemblent les résultats de ces expériences.

Le tableau I indique 1° la quantité d'azote ingérée, 2° la répartition de l'azote trouvé dans le corps de l'estomac entre les protéines non attaquées et les divers groupes de produits de leur digestion, 3° les quantités d'azote ammoniacal et d'azote aminé aliphatique existant dans cette partie de l'estomac. Le tableau II a trait à la répartition de l'azote de la région prépylorique de cet organe entre les protéines intactes et leurs principaux dérivés ainsi qu'aux quantités totales d'azote ammoniacal et d'azote aminé aliphatique y renfermées. Le tableau III donne ces mêmes indications pour l'ensemble de l'estomac et mentionne, en outre, la quantité d'azote ingéré disparue de cet organe au bout du laps de temps considéré. Pour les chiens H à J, sacrifiés 8 à 10 heures après le repas, on a dû se borner à examiner le contenu de l'estomac total, le corps de l'estomac ne renfermant plus de bol

[1] Ou plutôt des protéoses précipitées par demi-saturation au moyen du sulfate de zinc en milieu acide.

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TABLEAU I.

Chien en expérience	Poids de l'animal, en grammes	Durée de la digestion, en heures	Quantité de viande ingérée, en grammes	Azote ingéré, en grammes	Azote trouvé dans le corps de l'estomac — en grammes — à l'état coagulable	à l'état incoagulable — sous forme d'acid-albumine	sous forme de protéoses — hétéroalbumose et protoalbumose	autres protéoses	total	sous forme d'autres produits — précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total	total	total	en milligrammes — à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique
A	4300	1	107·5	3·7237	2·3012	0·0026	0·2742	0·2460	0·3589	0·0132	0·0248	0·0380	0·5608	2·8620	1·03	21·90
B	3200	2	80	2·9992	1·6982	0·0009	0·1708	0·1881	0·5202	0·0167	0·0394	0·0561	0·4159	2·1141	10·35	29·47
C	13200	3	330	11·0385	5·0346	0·0008	0·2025	0·0712	0·2737	0·0270	0·0082	0·0352	0·3097	5·3443	5·90	7·56
D	8600	4	215	8·7290	2·5620	0·0706	0·3820	0·4150	0·7970	0·1397	0·0178	0·1575	1·0251	3·5871	0·29	46·86
E	7500	5	187·5	7·5300	1·1290	0·0015	0·1962	0·6089	0·8051	0·1102	0·0580	0·1682	0·9748	2·1038	2·63	52·76
F	9700	6	242·5	8·0850	0·3334	0·2140	0·3159	0·3202	0·6361	0·0548	0·0887	0·1425	0·9936	1·3270	16·86	16·10
G	12850	7	321·25	13·0620	0·2217	0·0029	0·1869	0·1559	0·3428	0·0082	0·0169	0·0251	0·3708	0·5925	12·04	9·15
H	13500	8	337·5	12·7845	—	—	—	—	—	—	—	—	—	—	—	—
I	5700	9	142·5	5·3879	—	—	—	—	—	—	—	—	—	—	—	—
J	9300	10	232·5	8·1561	—	—	—	—	—	—	—	—	—	—	—	—
K	10200	11	255	9·1339	—	—	—	—	—	—	—	—	—	—	—	—
L	9850	12	246·25	8·9079	—	—	—	—	—	—	—	—	—	—	—	—

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TABLEAU II.

Chien en expérience	Durée de la digestion, en heures	Azote trouvé dans la région prépylorique de l'estomac — en grammes — à l'état coagulable	à l'état incoagulable — sous forme d'acid-albumine	sous forme de protéoses — hétéroalbumose et protoalbumose	autres protéoses	total	sous forme d'autres produits — précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total	total	total	en milligrammes — à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique
A	1	0·1326	0·0002	0·0230	0·0247	0·0477	0·0082	0·0196	0·0278	0·0757	0·2083	1·11	4·26
B	2	0·1233	0·0000	0·0155	0·0299	0·0454	0·0016	0·0297	0·0313	0·0767	0·2000	4·52	6·59
C	3	0·1576	0·0002	0·0208	0·0126	0·0334	0·0846	0·0345	0·1191	0·1527	0·3103	5·60	4·78
D	4	0·2358	0·0110	0·0445	0·0674	0·1119	0·0416	0·0201	0·0617	0·1846	0·4204	0·27	9·15
E	5	0·1905	0·0004	0·0056	0·0317	0·0373	0·0304	0·0558	0·0862	0·1239	0·3144	5·05	4·73
F	6	0·0370	0·0323	0·0438	0·0526	0·0964	0·0071	0·0344	0·0415	0·1702	0·2072	6·72	7·60
G	7	0·1786	0·0006	0·0246	0·0544	0·0790	0·0105	0·0359	0·0464	0·1260	0·3046	4·50	11·65

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TABLEAU III.

Chien en expérience	Durée de la digestion, en heures	Azote trouvé dans l'estomac total — en grammes — à l'état coagulable	à l'état incoagulable — sous forme d'acid-albumine	sous forme de protéoses — hétéroalbumose et protoalbumose	autres protéoses	total	sous forme d'autres produits — précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total	total	total	en milligrammes — à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique	Azote disparu de l'estomac, en grammes
A	1	2·4338	0·0028	0·2972	0·2707	0·5679	0·0214	0·0444	0·0658	0·6365	3·0703	2·14	26·16	0·6534
B	2	1·8215	0·0009	0·1863	0·2180	0·4043	0·0183	0·0697	0·0874	0·4926	2·3141	14·87	36·06	0·6851
C	3	5·1912	0·0010	0·2233	0·0838	0·3071	0·1116	0·0427	0·1543	0·4624	5·6546	11·50	12·34	5·3839
D	4	2·7978	0·0816	0·4265	0·4824	0·9089	0·1813	0·0379	0·2192	1·2097	4·0075	0·56	56·01	4·7225
E	5	1·3195	0·0019	0·2018	0·6406	0·8424	0·1406	0·1138	0·2544	1·0987	2·4182	7·68	57·49	5·1118
F	6	0·3704	0·2463	0·3597	0·3728	0·7325	0·0619	0·1231	0·1850	1·1638	1·5342	23·58	23·70	6·5508
G	7	0·4003	0·0035	0·2115	0·2103	0·4218	0·0187	0·0528	0·0715	0·4968	0·8971	16·54	20·80	12·1649
H	8	0·1384	0·0479	0·1676	0·3036	0·4712	0·0352	0·0828	0·1180	0·6371	0·7755	25·76	8·25	12·0090
I	9	0·0108	0·0003	0·0436	0·0892	0·1328	0·0395	0·0238	0·0633	0·1964	0·2072	7·85	4·69	5·1807
J	10	0·0367	0·0068	0·0604	0·0719	0·1323	0·0167	0·0259	0·0426	0·1817	0·2184	4·62	2·28	7·9377

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alimentaire ou à peine. Quant aux chiens K et L, sacrifiés respectivement 11 et 12 heures après le repas, leurs estomacs étaient entièrement vides. Le tableau IV relate les chiffres d'azote trouvés chez les chiens A à J dans la première portion de l'intestin grêle à l'état d'acidalbumine ou d'autres dérivés des protéines, d'azote ammoniacal et d'azote aminé aliphatique.

On a calculé les diverses données des tableaux I à III en pour-cent de l'azote ingéré (tableau V) et en pour-cent de l'azote contenu dans la région de l'estomac examinée (tableau VI), celles des tableaux I à IV en pour-cent de l'azote incoagulable de la région du tube digestif envisagée (tableau VII).

TABLEAU IV.

		Azote trouvé dans la première portion de l'intestin grêle à l'état incoagulable									
		en grammes								en milligrammes	
		sous forme d'acid-albumine	sous forme de protéoses			sous forme d'autres produits			total		
Chien en expérience	Durée de la digestion, en heures		hétéroalbumose et protoalbumose	autres protéoses	total	précipités par l'acide phosphotungstique	non précipités par l'acide phospho-tungstique	total		à l'état d'azote ammo-niacal	à l'état d'azote aminé aliphatique
A	1	0·0000	0·0143	0·0191	0·0334	0·0672	0·0224	0·0896	0·1230	10·38	13·14
B	2	0·0000	0·0024	0·0022	0·0046	0·0140	0·0234	0·0374	0·0420	3·15	7·20
C	3	0·0000	0·0037	0·0042	0·0079	0·0745	0·1287	0·2032	0·2111	10·24	10·59
D	4	0·0000	0·0023	0·0035	0·0058	0·0651	0·0436	0·1087	0·1145	3·67	16·78
E	5	0·0006	0·0070	0·0051	0·0121	0·0610	0·1123	0·1733	0·1860	13·49	36·62
F	6	0·0000	0·0261	0·0282	0·0543	0·0569	0·0736	0·1305	0·1848	13·65	40·97
G	7	0·0000	0·0202	0·0416	0·0618	0·0405	0·0506	0·0911	0·1529	5·88	32·63
H	8	0·0000	0·0046	0·0048	0·0094	0·0243	0·0541	0·0784	0·0878	5·77	25·42
I	9	traces	0·0012	0·0087	0·0099	0·0293	0·0202	0·0495	0·0594	2·40	5·00
J	10	0·0000	0·0124	0·0327	0·0451	0·0736	0·0339	0·1075	0·1526	13·24	27·62

De plus, dans ce dernier tableau, on a établi la moyenne des résultats obtenus dans cette série d'expériences.

On n'a pas mentionné dans les tableaux I à IV les résultats donnés par la réaction du biuret dans les liquides privés des protéines, de l'acidalbumine et des protéoses, car elle n'a jamais fait défaut. Par conséquent, les deux régions de l'estomac et la première portion de l'intestin grêle contenaient toujours des peptones vraies de *Kühne*.

Comme dans toutes les séries d'expériences relatives à la digestion gastrique des protéines, on retrouve de moins en moins d'azote dans l'estomac au fur et à mesure qu'on s'éloigne du repas. Conformément aux résultats des

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TABLEAU V.

Chien en expérience	Durée de la digestion, en heures	Portion de l'estomac examinée	Pour-cent de l'azote ingéré retrouvé dans l'estomac												Disparus de l'estomac
			à l'état coagulable	à l'état incoagulable								total			
				sous forme d'acid-albumine	sous forme de protéoses			sous forme d'autres produits			total				
					hétéroalbumose et protoalbumose	autres protéoses	total	précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total			à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique	
A	1	corps	61·80	0·07	7·36	6·61	13·97	0·36	0·66	1·02	15·06	76·86	0·03	0·59	
		région prépylorique	3·56	0·01	0·62	0·66	1·28	0·22	0·52	0·74	2·03	5·59	0·03	0·11	
		estomac total	65·36	0·08	7·98	7·27	15·25	0·58	1·18	1·76	17·09	82·45	0·06	0·70	17·55
B	2	corps	56·62	0·03	5·70	6·27	11·97	0·55	1·32	1·87	13·87	70·49	0·35	0·98	
		région prépylorique	4·11	0·00	0·52	1·00	1·52	0·05	0·99	1·04	2·56	6·67	0·15	0·22	
		estomac total	60·73	0·03	6·22	7·27	13·49	0·60	2·31	2·91	16·43	77·16	0·50	1·20	22·84
C	3	corps	45·61	0·01	1·83	0·65	2·48	0·24	0·08	0·32	2·81	48·42	0·05	0·07	
		région prépylorique	1·48	traces	0·19	0·11	0·30	0·77	0·31	1·08	1·38	2·81	0·05	0·04	
		estomac total	47·09	0·01	2·02	0·76	2·78	1·01	0·39	1·40	4·19	51·23	0·10	0·11	48·77
D	4	corps	29·35	0·81	4·38	4·75	9·13	1·60	0·20	1·80	11·74	41·09	traces	0·54	
		région prépylorique	2·70	0·13	0·51	0·77	1·28	0·48	0·23	0·71	2·12	4·82	traces	0·10	
		estomac total	32·05	0·94	4·89	5·52	10·41	2·08	0·43	2·51	13·86	45·91	traces	0·64	54·09
E	5	corps	14·99	0·02	2·61	8·08	10·69	1·47	0·77	2·24	12·95	27·94	0·03	0·70	
		région prépylorique	2·53	0·01	0·07	0·42	0·49	0·40	0·74	1·14	1·64	4·17	0·07	0·06	
		estomac total	17·52	0·03	2·68	8·50	11·18	1·87	1·51	3·38	14·59	32·11	0·10	0·76	67·89
F	6	corps	4·13	2·65	3·91	3·96	7·87	0·68	1·09	1·77	12·29	16·42	0·20	0·20	
		région prépylorique	0·46	0·40	0·54	0·65	1·19	0·09	0·42	0·51	2·10	2·56	0·08	0·09	
		estomac total	4·59	3·05	4·45	4·61	9·06	0·77	1·51	2·28	14·39	18·98	0·28	0·29	81·02
G	7	corps	1·70	0·02	1·43	1·19	2·62	0·07	0·13	0·20	2·84	4·54	0·09	0·07	
		région prépylorique	1·37	0·01	0·19	0·41	0·60	0·08	0·27	0·35	0·96	2·33	0·04	0·09	
		estomac total	3·07	0·03	1·62	1·60	3·22	0·15	0·40	0·55	3·80	6·87	0·13	0·16	93·13
H	8	estomac total	1·08	0·37	1·31	2·38	3·69	0·27	0·65	0·92	4·98	6·06	0·20	0·06	93·94
I	9	estomac total	0·20	0·01	0·81	1·65	2·46	0·74	0·44	1·18	3·65	3·85	0·15	0·09	96·15
J	10	estomac total	0·45	0·09	0·74	0·88	1·62	0·20	0·32	0·52	2·23	2·68	0·06	0·03	97·32

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TABLEAU VI.

Chien en expérience	Durée de la digestion, en heures	Portion de l'estomac examinée	Pour-cent de l'azote contenu dans la région de l'estomac examinée — en grammes — à l'état coagulable	à l'état incoagulable — sous forme d'acid-albumine	sous forme de protéoses — hétéroalbumose et protoalbumose	autres protéoses	total	sous forme d'autres produits — précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total	total	en milligrammes — à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique
A	1	corps	80·41	0·09	9·58	8·59	18·17	0·46	0·87	1·33	19·59	0·04	0·76
		région prépylorique	63·66	0·09	11·04	11·86	22·90	3·94	9·41	13·35	36·34	0·53	2·05
		estomac total	79·27	0·09	9·68	8·82	18·50	0·70	1·44	2·14	20·73	0·07	0·85
B	2	corps	80·33	0·04	8·08	8·90	16·98	0·79	1·86	2·65	19·67	0·49	1·39
		région prépylorique	61·65	0·00	7·75	14·95	22·70	0·80	14·85	15·65	38·35	2·26	3·30
		estomac total	78·71	0·04	8·05	9·42	17·47	0·79	2·99	3·78	21·29	0·64	1·56
C	3	corps	94·20	0·01	3·79	1·33	5·12	0·52	0·15	0·67	5·80	0·11	0·15
		région prépylorique	50·79	0·06	6·70	4·06	10·76	27·27	11·12	38·39	49·21	1·80	1·54
		estomac total	91·82	0·02	3·95	1·48	5·43	1·97	0·76	2·73	8·18	0·20	0·22
D	4	corps	71·42	1·97	10·65	11·57	22·22	3·89	0·50	4·39	28·58	0·01	1·31
		région prépylorique	56·09	2·61	10·59	16·03	26·62	9·90	4·78	14·68	43·91	0·06	2·18
		estomac total	69·82	2·04	10·64	12·04	22·68	4·52	0·95	5·47	30·10	0·01	1·40
E	5	corps	53·66	0·07	9·33	28·94	38·27	5·24	2·76	8·00	46·34	0·12	2·51
		région prépylorique	60·59	0·13	1·78	10·08	11·86	9·68	17·74	27·42	39·41	1·57	1·50
		estomac total	54·56	0·08	8·35	26·49	34·84	5·81	4·71	10·52	45·44	0·32	2·38
F	6	corps	25·12	16·13	23·81	24·13	47·94	4·12	6·69	10·81	74·88	1·27	1·21
		région prépylorique	17·86	15·59	21·14	25·38	46·52	3·43	16·60	20·03	82·14	3·24	3·67
		estomac total	24·14	16·05	23·45	24·30	47·75	4·04	8·02	12·06	75·86	1·54	1·56
G	7	corps	37·42	0·49	31·55	26·31	57·86	1·38	2·85	4·23	62·58	2·03	1·54
		région prépylorique	58·63	0·20	8·08	17·86	25·94	3·45	11·78	15·23	41·37	1·48	3·82
		estomac total	44·62	0·39	23·78	23·44	47·82	2·08	5·89	7·97	55·38	1·84	2·32
H	8	estomac total	17·85	6·17	21·61	39·15	60·76	4·54	10·68	15·22	82·15	3·32	1·06
I	9	estomac total	5·21	0·15	21·04	43·05	64·09	19·06	11·49	30·55	94·79	3·79	2·26
J	10	estomac total	16·80	3·11	27·65	32·93	60·58	7·65	11·86	19·51	83·20	2·12	1·04

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TABLEAU VII.

Chien en expérience	Durée de la digestion, en heures	Portion du tube digestif examinée	Pour-cent de l'azote incoagulable contenu dans la région du tube digestif examinée: sous forme de protéoses: sous forme d'acidalbumine	hétéroalbumose et protoalbumose	autres protéoses	total	sous forme d'autres produits: précipités par l'acide phosphotungstique	non précipités par l'acide phosphotungstique	total	à l'état d'azote ammoniacal	à l'état d'azote aminé aliphatique
A	1	corps de l'estomac	0·46	48·90	43·86	92·76	2·36	4·42	6·78	0·18	3·91
		région prépylorique	0·26	30·38	32·63	63·01	10·84	25·89	36·73	1·47	5·63
		estomac total	0·44	46·68	42·54	89·22	3·36	6·98	10·34	0·34	4·11
		intestin grêle	0·00	11·62	15·53	27·15	54·64	18·21	72·85	8·44	10·68
B	2	corps de l'estomac	0·22	41·07	45·22	86·29	4·02	9·47	13·49	2·49	7·09
		région prépylorique	0·00	20·21	38·98	59·19	2·09	38·72	40·81	5·89	13·65
		estomac total	0·19	37·82	44·25	82·07	3·71	14·03	17·74	3·08	7·45
		intestin grêle	0·00	5·71	5·24	10·95	33·33	55·72	89·05	7·50	17·14
C	3	corps de l'estomac	0·26	65·39	22·99	88·38	8·71	2·65	11·36	1·91	2·44
		région prépylorique	0·13	13·62	8·25	21·87	55·40	22·60	78·00	3·67	3·13
		estomac total	0·22	48·29	18·12	66·41	24·14	9·23	33·37	2·49	2·67
		intestin grêle	0·00	1·75	1·99	3·74	35·29	60·97	96·26	4·85	5·02
D	4	corps de l'estomac	6·89	37·26	40·48	77·74	13·63	1·74	15·37	0·03	4·57
		région prépylorique	5·96	24·11	36·51	60·62	22·53	10·89	33·42	0·15	4·96
		estomac total	6·75	35·25	39·88	75·13	14·99	3·13	18·12	0·05	4·63
		intestin grêle	0·00	2·01	3·06	5·07	56·85	38·08	94·93	3·21	14·65
E	5	corps de l'estomac	0·15	20·13	62·46	82·59	11·30	5·96	17·26	0·27	5·41
		région prépylorique	0·32	4·52	25·58	30·10	24·54	45·04	79·58	4·08	3·82
		estomac total	0·17	18·37	58·30	76·67	12·80	10·36	23·16	0·70	5·23
		intestin grêle	0·32	3·76	2·74	6·50	32·80	60·38	93·18	7·25	19·69
F	6	corps de l'estomac	21·54	31·79	32·23	64·02	5·41	8·93	14·44	1·70	1·62
		région prépylorique	18·98	25·73	30·91	56·64	4·17	20·21	24·38	3·95	4·47
		estomac total	21·16	30·91	32·03	62·94	5·32	10·58	15·90	2·03	2·04
		intestin grêle	0·00	14·12	15·26	29·38	30·79	39·83	70·62	7·39	22·17
G	7	corps de l'estomac	0·78	50·41	42·04	92·45	2·21	4·56	6·77	3·25	2·47
		région prépylorique	0·48	19·52	43·18	62·70	8·33	28·49	36·82	3·57	9·01
		estomac total	0·70	42·57	42·34	84·91	3·76	10·63	14·39	3·33	4·19
		intestin grêle	0·00	13·21	27·21	40·42	26·49	33·09	59·58	3·85	21·35
H	8	estomac total	7·52	26·30	47·66	73·96	5·52	13·00	18·52	4·04	1·29
		intestin grêle	0·00	5·24	5·47	10·71	27·67	61·62	89·29	6·57	28·82
I	9	estomac total	0·15	22·20	45·42	67·62	20·11	12·12	32·23	4·00	2·39
		intestin grêle	0·00	8·13	21·42	29·55	22·22	48·23	70·45	8·68	18·10
J	10	estomac total	3·74	33·24	39·57	72·81	9·19	14·26	23·45	2·54	1·25
		intestin grêle	0·00	2·02	14·64	16·66	49·33	34·01	83·34	4·01	8·40
moyenne générale des résultats		corps de l'estomac	4·33	42·14	41·32	83·46	6·81	5·40	12·21	1·40	3·93
		portion prépylorique	3·73	19·72	30·87	50·59	18·27	27·41	45·68	3·25	7·38
		estomac total	4·10	34·17	41·01	75·18	10·29	10·43	20·72	2·26	3·53
		intestin grêle	0·03	6·76	11·25	18·01	36·94	45·02	81·96	6·17	16·60

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recherches antérieures, la digestion a progressé plus vite pendant les trois premières heures consécutives à l'ingestion de la viande cuite de bœuf qu'ensuite. Après la septième heure, l'évacuation de l'estomac n'a plus que progressé très lentement. L'achèvement de la digestion gastrique a demandé 10 à 11 heures au lieu d'un peu plus de 8 lorsqu'il s'est agi de la viande cuite de cheval.

Une fois de plus, la présente série d'expériences vérifie que la digestion gastrique de la viande cuite s'effectue plus vite, chez le chien, pendant les premières heures du processus digestif qu'ensuite, si l'on se base sur l'azote disparu de l'estomac, l'azote coagulable et l'azote incoagulable trouvés dans ce viscère.

L'azote coagulable tend à diminuer graduellement avec le laps de temps écoulé depuis le repas, tant dans le corps de l'estomac que dans l'ensemble de cet organe. Cette diminution est moins nette dans la portion prépylorique de l'estomac et ne s'observe guère qu'à partir de la troisième heure après l'ingestion de viande cuite de bœuf.

L'estomac ne renferme que très peu d'acidalbumine, sauf chez les chiens D, F et H. On en trouve une moindre proportion dans la portion prépylorique de cet organe que dans le corps de l'estomac. Toutefois, chez le chien E, la proportion d'azote incoagulable à l'état d'acidalbumine l'emporte dans la région prépylorique de l'estomac. Cette portion de cet organe ne renferme pas du tout d'acidalbumine chez le chien B et en contient à peine chez les chiens A, C, E et G. Sauf chez le chien E, il n'existe pas d'acidalbumine, ou tout au plus des traces (chien I) dans la première portion de l'intestin grêle.

La proportion d'azote incoagulable à l'état de protéoses diminue du corps de l'estomac à la portion prépylorique de cet organe et surtout de celle-ci à l'intestin grêle. Tel est toujours le cas pour les protéoses précipitées par demi-saturation au moyen du sulfate de zinc. Il en est de même pour les autres albumoses, sauf chez les chiens F et G, chez lesquels les deux régions renferment à peu près la même proportion de ces produits.

Au contraire la proportion d'azote incoagulable à l'état de peptones et de composés abiurétiques s'accroît du corps de l'estomac à la région prépylorique de cet organe et de celle-ci à l'intestin grêle. Il en est constamment ainsi pour les produits précipités par l'acide phosphotungstique, à l'exception des chiens B et F, chez lesquels la portion prépylorique de l'estomac présente une teneur en ces composés moins élevée que le corps de l'estomac, et du chien C, chez lequel la région prépylorique contient une proportion plus considérable de ces composés que les deux autres parties du tube digestif examinées.

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La proportion d'azote incoagulable à l'état de composés non précipités par l'acide phosphotungstique augmente du corps de l'estomac à la région prépylorique de cet organe et de celle-ci à l'intestin grêle, sauf chez le chien A, chez lequel la teneur en ces composés est plus grande dans la région prépylorique de l'estomac que dans l'intestin.

Les protéoses non précipitées par demi-saturation au moyen du sulfate de zinc l'emportent sur les autres quatre fois sur sept dans le contenu du corps de l'estomac, six fois sur sept dans la portion prépylorique de l'estomac, sept fois sur dix dans l'estomac total, huit fois sur dix dans l'intestin. La quantité d'azote incoagulable à l'état d'hétéroalbumose et de protoalbumose est, en réalité, à peu près la même que celle sous forme des autres protéoses dans le contenu de l'estomac du chien G et de l'intestin des chiens B et H.

Les peptones et les composés abiurétiques non précipités par l'acide phosphotungstique prédominent sur les produits abiurétiques précipités par ce réactif, sauf dans le corps de l'estomac chez les chiens C, D et E, dans la portion prépylorique de cet organe chez les chiens C et D, dans l'estomac total chez les chiens C, D, E et I, dans l'intestin grêle chez les chiens A, D et J.

Le tableau VIII permet de comparer la répartition de l'azote non coagulable du contenu de l'estomac et de celui de la première portion de l'intestin grêle entre l'acidalbumine, les protéoses et les autres produits de scindage des protéines après l'ingestion de viande cuite de bœuf, selon qu'on emploie la méthode par filtration ou celle par centrifugation.

TABLEAU VIII.

Portion du tube digestif examinée	Méthode expérimentale employée	Pour-cent de l'azote non coagulable sous forme					
		d'acidalbumine		de protéoses		d'autres produits	
		chiffres extrêmes	moyenne	chiffres extrêmes	moyenne	chiffres extrêmes	moyenne
estomac total	filtration		traces	78·94 à 90·94	88·39	9·06 à 21·06	11·61
	centrifugation	0·15 à 21·16	4·10	62·94 à 89·22	75·18	10·34 à 33·37	20·72
première portion de l'intestin grêle	filtration			9·86 à 29·00	17·16	71·00 à 90·14	82·84
	centrifugation	0·00 à 0·32	0·03	3·74 à 40·42	18·01	59·58 à 96·26	81·96

Les chiffres relatifs à la méthode par filtration mentionnés dans ce tableau proviennent des multiples expériences effectuées antérieurement de cette façon [Zunz, 1910,2]. Il n'est question dans ce tableau que de l'estomac total et de la première portion de l'intestin grêle, parce que dans les diverses

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expériences effectuées jusqu'à présent en séparant le contenu du corps de l'estomac de celui de la région prépylorique de cet organe, on n'a jamais fait ingérer des chiens de la viande cuite de bœuf. En présence des avantages de la méthode par centrifugations successives, il n'y a pas lieu de se préoccuper de combler cette lacune.

Pour l'estomac considéré dans son ensemble, on constate moins de protéoses, davantage d'acidalbumine d'une part ainsi que de peptones et de composés abiurétiques d'autre part, au moyen de la méthode par centrifugation qu'au moyen de celle par filtration. Pour la première portion de l'intestin grêle, les chiffres moyens obtenus pour la répartition de l'azote incoagulable sont les mêmes, quel que soit le procédé employé: pas d'acidalbumine ou seulement des traces, moins du cinquième à l'état de protéoses, plus des $\frac{4}{5}$ à l'état de peptones et surtout de composés abiurétiques.

Les peptones et les corps abiurétiques ne dépassent guère le cinquième de l'azote incoagulable dans l'estomac total lorsqu'on procède par filtrations successives. Ces composés peuvent représenter jusqu'au tiers de cet azote lorsqu'on opère par centrifugations successives. Ces dérivés des protéines l'emportent toujours sur les autres produits de la désintégration des substances albuminoïdes dans la première portion de l'intestin grêle, quelle que soit la méthode expérimentale employée.

Occupons nous maintenant du problème que nous nous étions posés, à savoir si l'on observe, lors de la digestion de la viande cuite de bœuf, les mêmes différences entre les résultats des procédés par filtrations et par centrifugations successives que lors de la digestion de la viande cuite de cheval.

La proportion d'azote incoagulable à l'état d'acidalbumine est un peu plus élevée, tout en restant relativement faible, dans les deux régions de l'estomac aussi bien après l'ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval lorsqu'on utilise la centrifugation que lorsqu'on emploie la filtration. Dans les deux cas, l'intestin ne renferme pas ou guère d'acidalbumine.

Ce parallélisme des résultats ne s'observe plus pour les protéoses et les autres dérivés des protéines. Après l'ingestion de viande cuite de cheval, la proportion d'azote incoagulable à l'état de protéoses est à peu près la même par les deux procédés dans le corps de l'estomac et dans l'ensemble de cet organe. Elle est un peu plus considérable lorsqu'on utilise la méthode par centrifugation dans la région prépylorique de l'estomac et dans la première portion de l'intestin grêle. Par contre, la proportion d'azote incoagulable à

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l'état de peptones et de composés abiurétiques (polypeptides pour l'estomac, en outre acides aminés pour l'intestin), précipités ou non par l'acide phosphotungstique est un peu inférieure dans le corps de l'estomac par la méthode de la centrifugation que par celle de la filtration. Ce phénomène s'accentue davantage dans la portion prépylorique de l'estomac et encore plus dans la première partie de l'intestin grêle.

L'influence de la méthode utilisée pour la séparation des diverses espèces de dérivés des protéines n'est donc en aucune façon la même pour l'estomac total selon que le chien ingère de la viande cuite de cheval ou de bœuf. Elle est nulle dans l'intestin après l'ingestion de viande cuite de bœuf. Il est impossible pour le moment de reconnaître à quoi sont dues les différences signalées ci-dessus entre les résultats obtenus par les méthodes de filtration et de centrifugation selon l'espèce de viande ingérée.

TABLEAU IX.

Portion du tube digestif examinée	Espèce de viande cuite ingérée	Pour-cent de l'azote non coagulable en moyenne sous forme		
		d'acidalbumine	de protéoses	d'autres produits
corps de l'estomac	bœuf	4·33	83·46	12·21
	cheval	2·84	76·71	20·45
portion prépylorique de l'estomac	bœuf	3·73	50·59	45·68
	cheval	1·43	67·40	31·17
estomac total	bœuf	4·10	75·18	20·72
	cheval	2·35	37·31	20·34
première portion de l'intestin grêle	bœuf	0·03	18·01	81·96
	cheval	0·00	30·75	69·25

Quoi qu'il en soit, il est certain que le procédé par centrifugations successives est à l'abri de plusieurs causes d'erreur inhérentes à la méthode par filtrations successives.

Que ressort il du tableau IX destiné à comparer la répartition moyenne de l'azote non coagulable des contenus des diverses régions du tube digestif entre l'acidalbumine, les protéoses et les autres dérivés des protéines, selon l'espèce de viande cuite ingérée?

Remarquons avant tout que les données du tableau IX, comme du reste aussi celles du tableau X, relatives à l'estomac considéré dans son ensemble et à la première partie de l'intestin grêle représentent les moyennes d'un plus grand nombre de résultats que les chiffres ayant trait au corps de l'estomac et à la portion prépylorique de cet organe.

Il y a partout davantage d'acidalbumine après ingestion de viande cuite

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de bœuf qu'après celle de viande cuite de cheval. Il y a davantage de protéoses dans le corps de l'estomac et dans l'estomac total après ingestion de viande cuite de bœuf qu'après ingestion de viande cuite de cheval, moins au contraire dans la portion prépylorique de cet organe et dans l'intestin grêle. Quant aux autres dérivés des protéines (peptones et polypeptides pour l'estomac, acides aminés en outre pour l'intestin), on en observe une proportion moindre après ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval dans le corps de l'estomac, une proportion plus considérable dans la portion prépylorique de cet organe, dans l'ensemble de ce viscère et dans la première portion de l'intestin grêle.

La désintégration des protéines semble donc être poussée en moyenne un peu moins loin dans le corps de l'estomac après l'ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval, un peu plus loin dans la portion prépylorique de l'estomac et dans la première partie de l'intestin grêle.

Arrivons en à l'azote ammoniacal et à l'azote aminé aliphatique. Après l'ingestion de viande cuite de bœuf, ces deux espèces d'azote n'ont jamais fait défaut dans les trois régions du tube digestif examinées.

Les proportions d'azote ammoniacal et d'azote aminé aliphatique s'accroissent toujours du corps de l'estomac à la portion prépylorique de cet organe et de celle-ci à l'intestin. Toutefois, chez le chien G, l'accroissement de l'azote ammoniacal est insignifiant. En outre, chez le chien E, la teneur en azote aminé aliphatique de la portion prépylorique de l'estomac est inférieure à celle du corps de cet organe.

Le contenu intestinal présente toujours une teneur en azote aminé aliphatique supérieure à sa teneur en azote ammoniacal. Il en est aussi ainsi pour la portion prépylorique de l'estomac, à l'exception des chiens C et E. Tel est encore le cas pour le corps de l'estomac, sauf chez les chiens F et G, et pour le contenu total de l'estomac des chiens A, B, C, D, E et G. Chez le chien F, la teneur en azote ammoniacal et celle en azote aminé aliphatique sont les mêmes dans l'estomac considéré dans son ensemble. Chez les chiens H à J, sacrifiés huit à dix heures après le repas et dont l'estomac ne renferme plus qu'une faible quantité de bol alimentaire, l'azote ammoniacal l'emporte sur l'azote aminé dans ce viscère.

Le tableau X a pour but de comparer les teneurs en azote ammoniacal et en azote aminé aliphatique des diverses régions du tube digestif après ingestion de viande cuite, soit de bœuf, soit de cheval.

Si l'on se base sur l'azote incoagulable, la teneur du contenu des deux régions de l'estomac en azote ammoniacal est, en moyenne, moindre après

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TABLEAU X.

Portion du tube digestif examinée	Espèce de viande cuite ingérée	Azote ammoniacal, en pour-cent				Azote aminé aliphatique, en pour-cent			
		de l'azote incoagulable		de l'azote total		de l'azote incoagulable		de l'azote total	
		chiffres extrêmes	moyenne	chiffres extrêmes	moyenne	chiffres extrêmes	moyenne	chiffres extrêmes	moyenne
corps de l'estomac	bœuf	0·03 à 3·25	1·40	0·01 à 2·03	0·58	1·25 à 7·09	3·93	0·15 à 2·51	1·27
	cheval	0·72 à 5·64	2·66	0·08 à 2·33	0·77	0·00 à 4·23	1·67	0·00 à 0·85	0·47
portion prépylorique de l'estomac	bœuf	0·15 à 5·89	3·25	0·06 à 3·24	1·55	3·13 à 13·65	7·38	1·50 à 3·82	2·57
	cheval	1·32 à 7·96	3·69	0·56 à 3·51	2·10	1·23 à 10·79	5·38	0·55 à 4·80	3·02
estomac total	bœuf	0·05 à 4·04	2·21	0·01 à 3·79	1·38	1·29 à 7·45	3·53	0·22 à 2·38	1·46
	cheval	0·98 à 5·82	2·65	0·17 à 2·41	0·84	0·42 à 5·25	2·13	0·05 à 1·20	0·62
première portion de l'intestin grêle	bœuf	3·21 à 8·68	6·17			5·02 à 28·82	16·60		
	cheval	1·51 à 8·33	4·45			5·86 à 16·80	10·71		

ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval. Elle est, par contre, plus élevée dans le premier cas que dans le second pour ce qui concerne le contenu de la première portion de l'intestin grêle. On observe les mêmes résultats si l'on prend pour point de départ l'azote total, sauf pour la teneur moyenne en azote ammoniacal de l'ensemble de l'estomac. Celle-ci l'emporte après ingestion de viande cuite de bœuf sur le chiffre observé après ingestion de viande cuite de cheval. Ceci est dû au fait que, chez les trois chiens H à J, sacrifiés huit à dix heures après avoir mangé de la viande de bœuf et dont on n'a pu examiner que l'ensemble de l'estomac par suite du peu de contenu encore présent dans ce viscère à ces moments, il existe une proportion relativement considérable d'azote ammoniacal pour une proportion relativement faible d'azote coagulable.

La teneur moyenne en azote aminé aliphatique est plus élevée, par rapport à l'azote incoagulable, après ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval dans toutes les régions du tube digestif examinées. Il en est encore ainsi par rapport à l'azote total pour le contenu soit du corps de l'estomac, soit de tout cet organe, mais par contre plus pour le contenu de la portion prépylorique de ce viscère.

Comme dans les expériences effectuées avec la viande cuite de cheval, on ne parvient pas, chez les chiens ayant ingéré de la viande cuite de bœuf, à déceler de relations spéciales bien nettes entre la teneur en azote ammoniacal

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ou en azote aminé aliphatique des diverses régions du tube digestif et soit leur teneur en acidalbumine, en protéoses ou en autres dérivés de la désintégration des protéines, précipités ou non par l'acide phosphotungstique, soit le laps de temps écoulé depuis le repas. Néanmoins la teneur relativement considérable de la première portion de l'intestin grêle en azote ammoniacal et surtout en azote aminé aliphatique doit être rapprochée de la prédominance des peptones et des composés abiurétiques dans cette portion des voies digestives. Ceci est, d'ailleurs, aussi le cas après l'ingestion de viande cuite de cheval.

Résumé.

1. Après l'ingestion de viande cuite de bœuf, l'estomac renferme en moyenne, chez le chien, une plus forte proportion d'azote incoagulable à l'état de protéoses, une moindre proportion d'une part à l'état d'acidalbumine, d'autre part à l'état de peptones et de composés abiurétiques, lorsqu'on sépare les diverses espèces de dérivés des protéines par centrifugations successives que lorsqu'on utilise à cet effet l'ancienne méthode des filtrations successives. Au contraire les chiffres moyens obtenus pour la répartition de l'azote incoagulable du contenu de la première portion de l'intestin grêle sont les mêmes quelle que soit la méthode expérimentale employée: pas d'acidalbumine ou seulement des traces, moins du cinquième à l'état de protéoses, plus des $\frac{4}{5}$ à l'état de peptones et surtout de composés abiurétiques.

2. L'influence de la méthode utilisée pour la séparation des diverses espèces de dérivés des protéines n'est en aucune façon la même pour le contenu de l'estomac selon l'espèce de viande cuite ingérée. Elle est nulle pour le contenu intestinal après l'ingestion de viande cuite de bœuf.

3. On constate en moyenne davantage d'acidalbumine dans le contenu du corps de l'estomac, dans celui de la portion prépylorique de cet organe et dans celui de la première portion de l'intestin grêle après ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval. Il y a davantage de protéoses dans le corps de l'estomac et dans l'ensemble de cet organe après ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval, moins au contraire dans la portion prépylorique de l'estomac et dans l'intestin grêle. Quant aux autres dérivés des protéines (peptones et polypeptides pour l'estomac, acides aminés en outre pour l'intestin), on en observe une proportion moindre après ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval dans le corps de l'estomac, une proportion plus

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considérable par contre dans la portion prépylorique et dans l'ensemble de cet organe ainsi que dans la première partie de l'intestin grêle.

4. Tant la teneur en peptones et en composés abiurétiques que les teneurs en azote ammoniacal et en azote aminé aliphatique s'accroissent d'ordinaire du contenu du corps de l'estomac à celui de la portion prépylorique de cet organe et de celui-ci au contenu de la première partie de l'intestin grêle.

5. Il n'y a pas de relation bien nette entre le laps de temps écoulé depuis l'ingestion de viande cuite de bœuf par le chien et soit la répartition de l'azote incoagulable entre les divers groupes de dérivés des protéines, soit la teneur en azote ammoniacal ou en azote aminé aliphatique des diverses portions du tube digestif examinées.

6. La teneur moyenne en azote ammoniacal est moindre après ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval dans le contenu des deux régions de l'estomac, plus élevée par contre dans le contenu de la première portion de l'intestin grêle.

7. La teneur moyenne en azote aminé aliphatique est plus considérable dans le corps de l'estomac, dans la région prépylorique de cet organe et dans la première partie de l'intestin grêle après ingestion de viande cuite de bœuf qu'après celle de viande cuite de cheval.

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IV. A NOTE ON THE HOPKINS-COLE REACTION FOR PROTEIN.

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This work was undertaken at the suggestion of Dr Rothera, owing to the observation which had been made by those teaching in this laboratory and in the Physiology Laboratory, that this reaction usually failed with the acid supplied to the laboratories.

This, of course, was very disappointing to both students and teachers, as the reaction is generally thoroughly reliable, and moreover, very definite.

It was early observed that if old sulphuric acid were resorted to on encountering such failures, the reaction obtained was satisfactory.

Just as the work was commencing, Mottram's paper appeared in the *Biochemical Journal* [1914], and the work was then confined to seeking practical methods of improving the refractory acids. These invariably gave the brown or yellow-brown ring described by Mottram as being due to excess of oxidising agent.

Moreover, as no improvement was effected by the addition of traces of oxidising agents, such as $FeCl_3$, HNO_3, $NaNO_2$, it was very naturally suggested that excess of oxidising agent was already present. It was further thought that the old acids which gave the reaction might have lost some volatile impurity, such as oxides of nitrogen, and that these might be got rid of by mechanical means. However, attempts to obtain an improvement by heating the acid in a flat dish for an hour, or by bubbling air through the acid heated under low pressure for 86 hours, were unsuccessful.

The action of some reducing agents was then investigated, and, of those tried, the following were found to be the most useful.

1. *Magnesium.* The powdered metal used took about 24 hours to settle out sufficiently. The acid was then improved to some extent, the colour of

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the ring being a very dark brown-purple. It was not further improved by prolonged standing in contact with the metal.

2. *Sodium bisulphite.* The use of this salt appears to be the most ready means of improving the acid, but is rather wasteful on account of the relatively large amount needed. If the salt and acid are well mixed, the reaction will be given as soon as the acid has cleared, which generally takes about five minutes. The colour of the ring is a purple-violet, and acid treated in this way retained the improvement after three months.

3. *Zinc.* (*a*) *In powdered form.* The powder used was extremely fine, and it was some weeks before the acid was clear enough to perform the test satisfactorily. The acid then seemed to err on the side of having too little oxidising agent, as the colour given was a blue-lilac, quite unlike the violet colour usually obtained. On running some weak $NaNO_2$ solution on to the ring, it developed into the characteristic violet-purple.

(*b*) *In the granulated form.* This provides the most satisfactory method of dealing with large quantities of acid for practical purposes. The acid should be well mixed to ensure thorough contact with the metal, and left for two or three days. The ring obtained is a deep violet-purple of great intensity, and shows no sign of browning.

The reagents used throughout were 2 % egg-white solution and "reduced oxalic," prepared according to the directions of Hopkins and Cole [1901].

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V. ON INACTIVE LIPASE AND THE NATURE OF ITS CO-ENZYME.

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Some years ago the observation was made in this laboratory by Rosenheim [1910] that active pancreatic lipase, as contained in glycerol extracts of pig's pancreas, can be separated into two inactive fractions by mere filtration through filter paper. The insoluble moiety on the filter paper contains the thermolabile inactive enzyme, which is restored to its original activity by the addition of the filtrate. The thermostable substance contained in the latter was designated as the co-enzyme according to the current nomenclature. Rosenheim also described a convenient method by which inactive lipase may be obtained as a dry powder free from soluble proteoclastic and amyloclastic enzymes, electrolytes, fats and lipoids.

From preliminary experiments it was concluded that the co-enzyme dialyses readily, and seems to be soluble in dilute but not in absolute alcohol, and insoluble in ether. Its activity is lost on incineration of the active dialysate. Of other substances tested bile salts and glycerophosphates were also found to possess activating powers.

These observations were fully confirmed by Pekelharing [1912], who made the additional interesting statement that the ash of the co-enzyme solution produced intense lipoclasis when added to the inactive lipase, if it had previously been dissolved in hydrochloric acid. He ascribed to calcium ions the main rôle in this reaction, which he explained by the removal of fatty acid as an insoluble calcium soap. Pekelharing failed, however, to adduce proofs that calcium salts are present in any amount in the co-enzyme solution; further, the effect due to calcium salts seems to account only for a relatively small amount of the total activating power of the co-enzyme solution.

Rona and Bien [1914], whilst partially confirming Rosenheim's results, were unable to demonstrate the presence of the co-enzyme. These observers,

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however, employed a phosphate mixture as a medium, in order to regulate the hydrogen ion concentration during the reaction. As it will be shown in this communication that phosphates by themselves exert a powerful activating action on inactive lipase, it is not surprising that these authors failed to find the presence of a co-enzyme in their extracts.

The observations of J. Mellanby and Woolley [1914] refer to the lipase in pancreatic juice. It is obvious that in this case a separation of enzyme and co-enzyme by mere filtration is impossible as the enzyme is in a soluble condition as distinguished from the suspended insoluble state in which it occurs in pancreatic extracts. Mellanby and Woolley made an unsuccessful attempt to effect a separation by alcohol. The impossibility of separating the lipase of pancreatic juice into enzyme and co-enzyme by means of alcohol precipitation does not, however, necessarily imply that their separation is impossible by other means.

The present investigation was undertaken in order to study in the first instance the activating power of the inorganic constituents of the co-enzyme solution. The action of electrolytes on pancreatic lipase has already been the subject of many investigations. The results have, however, been rather contradictory. Thus, to mention only two recent investigations, Terroine [1910] working with pancreatic juice and olive oil found an accelerating influence of Na, K, Mg and Ba salts, whilst Ca salts had no action. On the other hand, Mellanby and Woolley [1914] deny the influence of electrolytes altogether. Quite apart from the influence of the varying degree of emulsification of the zymolytes employed (fats or esters) it is obvious that both pancreatic juice and glycerol extracts of pancreas contain electrolytes in varying amount. Terroine has indeed attempted to remove the electrolytes by dialysis, but found that the lipase of pancreatic juice was destroyed in this process, probably by the action of the proteoclastic enzyme. Whilst therefore pancreatic juice and extracts appear to be unsuitable for the study of the action of electrolytes on their enzymes, this objection does not hold for the inactive lipase preparations made by Rosenheim's method, which can be obtained free from electrolytes and soluble enzymes by careful washing with water.

Whilst I was able to show conclusively that the inorganic constituents, especially the phosphates, contribute largely to the activity of the co-enzyme solution, the fact was incidentally established that the pancreatic co-enzyme is of a complex nature and that organic substances also are concerned in the reaction.

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Experimental.

Preparation of inactive lipase.

2 kilos. of fresh pig's pancreas, freed as far as possible from visible fat, were minced and mixed with 4 litres of glycerol. The mixture was frequently stirred and usually allowed to stand for 24 hours. By straining through two layers of fine muslin, a turbid extract was obtained, which possessed considerable fat-splitting activity.

Portions of one litre of this extract were diluted with ten litres of distilled water and the milky fluid allowed to settle in tall cylinders in the ice box. As a rule a perfect separation is obtained at this stage, a white precipitate settling after 24 hours' standing from which the clear supernatant fluid can be easily siphoned off. The precipitate is again suspended in 10 litres of distilled water. Probably owing to the removal of the electrolytes the lipase remains now usually in a colloidal suspension, but by means of a centrifuge a separation can be easily effected.

Pekelharing recommends the addition of acetic acid until the fluid is distinctly acid to sensitive litmus paper. A very satisfactory separation is certainly produced by this modification, but there is considerable danger that trypsin and trypsinogen also are precipitated by the addition of acetic acid. I have always found that although a better yield and a better separation are obtained with the help of acetic acid, the resulting preparation is always less active lipoclastically and also possesses a marked proteoclastic activity when tested with congo-fibrin. The use of acetic acid is therefore not to be recommended when a highly active preparation is desired.

Three washings are usually sufficient to free the preparation from the co-enzyme. The flocculent precipitate is next suspended in an equal volume of alcohol and filtered through a Pukall's [1893] porous clay filter. The precipitate is washed on the filter with acetone and finally with ether. After this treatment it easily peels off in flakes from the filter and after drying in vacuo is obtained as a fine greyish-white powder.

Besides my own preparations I have used an inactive lipase which had been prepared over two years ago by the late Mr Myers-Ward and which was kindly put at my disposal by Dr Rosenheim. This preparation had not been so fully freed from co-enzyme as my own, but its activity after the addition of co-enzyme was somewhat higher than that of mine. It is interesting to note that this powder retained its potential energy although kept for two years under the ordinary conditions of the laboratory.

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Preparation of co-enzyme solutions.

The fluid decanted from the precipitate obtained on diluting pancreatic glycerol extract with water, when boiled, filtered and concentrated contains the co-enzyme of lipase free from proteoclastic and other enzymes. The large percentage of glycerol, however, was thought to be in the way for the purpose of my experiments and I therefore used watery extracts of pancreas prepared according to Rosenheim's directions. 500 g. of minced pig's pancreas were boiled up with 500 cc. of distilled water, the mixture kept hot for 30 minutes and filtered after cooling. 620 cc. of extract were obtained which were preserved under toluene. On keeping, a sediment gradually formed mainly consisting of tyrosine; as this sediment was found to be devoid of any activating power, the clear supernatant fluid was used for the experiments.

The acidity of 1 cc. of the different preparations of co-enzyme solution varied from 1·4–2·5 cc. N/10 KOH. This amount, which remained constant after incubation with olive oil emulsion, was always allowed for.

Estimation of lipoclastic activity.

An emulsion of olive oil was used as the zymolyte and the degree of lipoclasis was determined by incubating the mixture of the enzyme and zymolyte for a given time (usually 17–20 hours) and titrating the fatty acids liberated with N/10 KOH. Alcohol was added in sufficient amount to keep the percentage of the titrated solution at least at 50 % alcohol and phenolphthalein was used as an indicator.

In order to obtain concordant results it is important that certain precautions are observed in carrying out these experiments. A short description of the routine followed in this laboratory may therefore not be out of place.

(*a*) Preparation of the oil emulsion. The quality of the olive oil employed is of considerable importance. On testing the activity of a pancreatic extract on a series of various commercial olive oils, it was found that under identical conditions some oils were easily attacked whilst others were hardly hydrolysed at all. One of these resisting oils was further investigated and was found to contain about 10 % of Arachis oil, an oil which is very resistant to the action of pancreatic lipase. The oil used in this laboratory is described as "Huile d'olive vierge" J. L. Duret & Co.

Before preparing the emulsion, the amount of N/10 KOH necessary to neutralise the free acid present is estimated by titrating 5 cc. of olive oil[1] without the addition of alcohol, until a slight permanent pink reaction to phenolphthalein is reached. Not more than 0·5 cc. N/10 KOH for 5 cc. of olive oil should be required. The calculated amount having been added to 50 cc. of

[1] For the exact measurement of small quantities of oil, emulsion or glycerol extracts, glass syringes are used made by grinding a glass rod into 5 cc. pipettes of about 25 cm. length, graduated into 1/10 cc.

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oil (about 5 cc. N/10 KOH) a uniform emulsion is obtained by means of a glass syringe, the oil being alternately drawn up and discharged in a shallow glass dish. 2·5 cc. or 5·0 cc. of this emulsion, which should not be made too thick, were used for each experiment.

(*b*) 10 mg. of inactive lipase is sufficient to obtain a well-marked reaction. It was used in a 0·2 % or 1 % suspension in water, which is prepared by first grinding the dry inactive lipase in a small mortar with a few drops of water into a fine paste and gradually adding the necessary volume of water. The suspension was freshly prepared for each set of experiments and care must be taken not to allow it to settle before measuring off the necessary amounts.

(*c*) The order in which enzyme, co-enzyme and zymolyte are mixed is of importance. In my experiments the enzyme suspension was measured into a series of small flasks first, the co-enzyme (or solution to be tested) being added next. 2·5 or 5 cc. of oil emulsion were mixed with these substances by shaking each flask for exactly the same time (20 seconds), it having been found that irregular results are obtained, unless this precaution is observed. The addition of toluene during digestion is unnecessary, as the large percentage of oil prevents bacterial development.

In the following table are given the results of experiments, made at various times, with the same preparations of inactive lipase and co-enzyme. In the last column is given the activity of the mixture of inactive enzyme and co-enzyme expressed in cc. N/10 KOH, the acidity due to co-enzyme solution and that produced by the inactive lipase alone having been deducted.

	Inactive lipase (10 mg. in 1 cc. water) cc.	Co-enzyme cc.	Time of incubation, hours	N/10 KOH cc.	Activity
No. 1	1	–	17	0·5	7·1
	1	1	,,	7·6	
	1	–	19	0·9	7·6
	1	1	,,	8·5	
	1	–	,,	0·9	7·0
	1	1	,,	7·9	
No. 2	1	–	17	2·5	10·5
	1	1	,,	13·0	
	1	–	16	2·5	8·9
	1	1	,,	11·4	
	1	2	,,	13·1	10·6
	1	–	17	2·5	9·4
	1	1	,,	11·9	

In the preparation of No. 1, acetic acid had been used and the co-enzyme had been carefully removed. The preparation had a very low initial activity, but its activity after the addition of the co-enzyme solution was not as great as that of No. 2, which had been prepared by Mr Myers-Ward without the use of acetic acid. These results fully confirm Rosenheim's observations and prove that the lipase of glycerol extracts of pig's pancreas can be separated into an inactive lipase and a co-enzyme.

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The action of the constituents of the ash of the co-enzyme.

After incineration of the active dialysate of a co-enzyme solution, Rosenheim found the filtered ash solution devoid of any action on the inactive lipase. This result may have been due to the alkalinity of this solution. Pekelharing observed that the ash of the co-enzyme solution (not dialysed) if neutralised with hydrochloric acid, conferred a strong lipoclastic activity on the inactive lipase. No quantitative experiments, however, are described by Pekelharing. For the purpose of my experiments I carefully incinerated portions of 5 cc. of watery pancreas extracts in a platinum dish and dissolved the ash in a small volume of water, after adding a drop of methyl red and the necessary amount (0·2–1·0 cc.) of N/10 HCl. The solution was evaporated to dryness in order to remove any excess of hydrochloric acid and the residue was dissolved again in water. The clear solution was made up to 5 cc. and its action compared with that of the original co-enzyme solution.

5 cc. of a 0·2 % suspension of inactive lipase and 5 cc. of oil emulsion were used. Time of incubation: 17 hours. The necessary corrections (in cc. N/10 KOH) were: 1 cc. co-enzyme (*a*) 2·0; 1 cc. ash solution (*a*) 0·3; 1 cc. co-enzyme (*b*) 1·6; 1 cc. ash solution (*b*) 0·4. The acidity due to inactive lipase alone was 2·5 cc. N/10 KOH. The results are given in the following table.

		Degree of lipoclasis	
		Titrated value N/10 KOH	Corrected value N/10 KOH
1.	Co-enzyme (*a*) cc.	cc.	cc.
	1	12·2	7·7
	1	12·4	7·9
	Ash solution (*a*) cc.		
	1	10·3	7·5
	1	9·2	6·4
2.	Co-enzyme (*b*) cc.		
	1	13·3	9·2
	1	13·0	8·9
	Ash solution (*b*) cc.		
	1	11·2	8·3
	1	10·9	8·0

From these results it seems that the solution of the ash of the co-enzyme, when dissolved in hydrochloric acid, possesses an activating power nearly equal to that of the original co-enzyme.

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Dialysis experiments.

Two different preparations of the co-enzyme solution were dialysed, one through a parchment diffusion shell (Schleicher and Schüll) and one through a sheep's appendix according to W. Wiechowski [1907]. The dialysis against distilled water was continued for four days, until a complete absence of chlorides in the dialysate was observed. The dialysate and the contents of the dialysing membranes were concentrated to the original volume. 5 cc. of each of these solutions were incinerated as above described and the activity of the ash-solutions on inactive lipase compared with that of the original solutions. 5 cc. of olive oil emulsion and 10 mg. of inactive lipase in 5 cc. of water were used in each case. Time of incubation: 18 hours. The necessary corrections were made as before.

The results are given in the following table.

		Degree of lipoclasis	
	Dialysate cc.	Titrated value cc. N/10 KOH	Corrected value cc. N/10 KOH
1	1	10·9	7·6
	2	12·3	8·4
2	1	10·5	7·4
	2	14·7	9·7
	Ash solution of dialysate cc.		
1	1	9·8	6·8
	2	9·6	7·3
2	1	9·3	6·8
	2	11·8	9·0
	Dialysed solution cc.		
1	1	3·0	1·0
	2	3·7	1·4
2	1	2·5	0·7
	2	3·5	1·5
	Ash solution of dialysed solution cc.		
1	1	2·6	0·8
	2	3·2	1·3
2	1	2·0	0·2
	2	2·8	0·9

These results prove that nearly the whole of the activating substances passes through the dialysing membranes as stated by Rosenheim. The ash of the incinerated dialysate, if dissolved in hydrochloric acid, produces practically the same effect as the original dialysate.

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From these experiments it would appear that the activity of the co-enzyme solution is not lost by incineration. This conclusion is, however, not valid in its entirety as will be seen from the following experiments. It has been found in the course of some unpublished work carried out by the late Mr Myers-Ward in this laboratory that the co-enzyme solution could be separated into two fractions by means of absolute alcohol. Both of these fractions, the alcohol-soluble as well as the alcohol-insoluble fraction, were able to activate inactive lipase. It seemed therefore of interest, to investigate the action of the ash constituents of these fractions.

Fractionation experiments with alcohol.

10 cc. of watery extract of pancreas were poured into 90 cc. of boiling absolute alcohol. A flocculent precipitate formed, from which the alcoholic solution was decanted. On the addition of more boiling alcohol the precipitate became granular and was easily filtered off, washed with alcohol and ether, and dried in vacuo.

The alcoholic solution was evaporated to dryness and the residue again

	Degree of lipoclasis	
	Titrated value cc. N/10 KOH	Corrected value cc. N/10 KOH
Alcohol-soluble fraction of co-enzyme cc.		
1	6·5	3·3
1	6·0	2·8
Ash solution cc.		
1	3·2	0·6
1	3·0	0·4
Alcohol-insoluble fraction of co-enzyme cc.		
1	8·5	5·0
1	8·3	4·8
Ash solution cc.		
1	6·0	3·2
1	5·8	3·0
Original co-enzyme cc.		
1	13·0	9·1
Ash solution cc.		
1	9·0	6·2

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taken up with boiling alcohol. The insoluble part was added to the main insoluble precipitate.

Before use the alcohol was evaporated and the residue dissolved in 10 cc. water. The alcohol-insoluble fraction also was dissolved in 10 cc. water.

5 cc. of each of these solutions were incinerated as described above and the action of these several fractions on inactive lipase compared with that of the original co-enzyme solution.

10 mg. of inactive lipase, 5 cc. oil emulsion. Time of incubation: 19 hours. The following corrections (cc. N/10 KOH) were necessary for the various solutions: 1 cc. alcohol-soluble fraction 0·7; 1 cc. ash solution 0·1; 1 cc. alcohol-insoluble fraction 1·0; 1 cc. ash solution 0·3; 1 cc. original watery extract 1·4; 1 cc. ash solution 0·3; 10 mg. inactive lipase 2·5. The results are given in the table on p. 45.

It is evident from these results that both the alcohol-soluble and the alcohol-insoluble fraction of the co-enzyme are able to activate inactive lipase, the former, however, to a smaller degree. Moreover, the greatest part of its activating power is destroyed by incineration. It is clear, therefore, that organic substances as well as electrolytes are concerned in this reaction.

I had also at my disposal an alcoholic extract of a co-enzyme solution prepared by the late Mr Myers-Ward. This had been prepared two years ago from a large quantity of material and had been purified to such an extent that its residue was completely alcohol-soluble. The solution had a slight deposit which was filtered off. When tested on the same inactive lipase preparation as previously, it was found to have lost some of its activity. This may have been contained in the insoluble deposit which was unfortunately no longer available. For comparison the results obtained by Mr Myers-Ward are given together with my own in the following table. The necessary corrections have been made.

	Activity	
Alcohol-soluble fraction of co-enzyme, cc.	(1912)	(1914)
1	12·7	5·1
1	12·7	5·1
Ash solution of same, cc.		
1	2·2	1·1
1	2·5	1·4

As was to be expected, the percentage of ash of this alcohol-soluble fraction was very small, namely 0·09 %, and it seemed to consist to a large extent of phosphoric acid. This led me to the investigation of the action of phosphoric

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acid and phosphates on inactive lipase. Except for Rosenheim's statement [1910] that glycerophosphates activate inactive lipase, no attention seems to have been given up till now to the influence of phosphates on fat splitting. In this connection it is interesting to note that according to Plimmer and Scott [1908] the pancreas alone of all the tissues examined contains phosphoproteins, and that these substances disappear from the pancreas during the secretion of pancreatic juice and are transformed to the largest extent into water-soluble phosphates which appear in the pancreatic juice [Plimmer and Kaya, 1910].

Before proceeding to carry out experiments with phosphates on inactive lipase, I estimated quantitatively the percentage of inorganic and organic phosphorus compounds in the co-enzyme solution employed.

Total P was estimated in 5 cc. of the solution by Neumann's method. For the estimation of organic P, 15 cc. of the solution were precipitated with the same volume of magnesia mixture and P estimated in the filtrate. Inorganic P was estimated by difference. The following results were obtained: Total P: 0·36 %; organic P: 0·25 %; inorganic P: 0·11 % The total ash of the co-enzyme solution was 0·59 %.

Hence the percentages of P in the ash are:

Total P	61 %.
Organic P	42 %.
Inorganic P	19 %.

It will be seen that the ash contains a very large percentage of P, 42 % of which is present in the original solution as organic phosphorus compounds (nucleic acid, organic phosphates) and 19 % as inorganic phosphates.

The action of phosphates on inactive lipase.

The action of very dilute solutions of phosphoric acid was first examined. Increasing quantities of these were added to 5 cc. of a 0·2 % suspension of inactive lipase. After bringing up to equal volume with water and adding 5 cc. of oil emulsion the mixtures were incubated for 17 hours. The acidity due to the added phosphoric acid was allowed for. The results are given in the following table.

H_3PO_4 added cc.	Molecular concentration of H_3PO_4 in mixture	Degree of lipoclasis: Titrated value cc. N/10 KOH	Degree of lipoclasis: Corrected value cc. N/10 KOH
0·1 M/100	M/17000	2·6	0
0·5 ,,	M/1400	3·1	+0·4
1·0 ,,	M/700	9·3	+6·5
0·2 M/10	M/350	11·7	+8·6
0·5 ,,	M/140	1·6	−2·4
Inactive lipase		2·5	0
Inactive lipase + co-enzyme (1 cc.)		13·5	9·4

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These experiments show conclusively that very dilute phosphoric acid solutions are able to activate inactive lipase to a marked degree, whilst an inhibition is exerted if a certain limit is overstepped. I have not investigated how far this result is connected with the optimum of hydrogen ion concentration for pancreatic lipase, but in the following experiment mixtures of primary and secondary phosphates were employed which enabled me to judge roughly the hydrogen ion concentration of the digestion mixture.

A M/15 solution of primary potassium phosphate and a M/15 solution of secondary sodium phosphate were prepared according to Sörensen [1912]. 6 cc. of definite mixtures of these were added to the usual mixture of inactive lipase and oil emulsion. The mixtures were incubated for 19 hours. In the following table are given the composition of the various phosphate mixtures, their titration values against phenolphthalein and their hydrogen ion concentration together with the effect they produced on inactive lipase.

	Phosphate mixture		Titration value of 6 cc. in cc. N/10 KOH	P_H.	Degree of lipoclasis	
	Primary cc.	Secondary cc.			Titrated value cc. N/10 KOH	Corrected value cc. N/10 KOH
1.	10·0	0·0	3·6	(4·494)	6·3	1·8
2.	9·75	0·25	2·4	5·288	6·2	2·9
3.	9·0	1·0	1·7	5·906	7·1	4·5
4.	7·0	3·0	1·3	6·468	6·6	4·4
5.	5·0	5·0	0·8	6·813	8·6	9·6
6.	3·0	7·0	0·5	7·168	11·2	9·8
7.	1·0	9·0	0·2	7·731	11·3	10·2
8.	0·0	10·0	0·1 N/10 H_2SO_4	(9·182)	6·7	5·9
	Inactive lipase				0·9	—
	Inactive lipase + co-enzyme				8·5	7·6

It is evident that all these phosphate mixtures appear to activate lipase. The action is most marked when a mixture in the proportion of 3 : 7 or 1 : 9 of primary and secondary phosphates is used. This fact incidentally explains also the failure of Rona and Bien [1914] to detect the presence of the co-enzyme in their extracts, as its effect was hidden by that produced by the phosphate mixtures employed by them in order to regulate the hydrogen ion concentration of their mixtures.

How far the action of phosphates is due to a direct action on the enzyme itself or to the production of a favourable reaction (hydrogen ion concentration) for its action, must be left for future investigations.

The action of calcium salts on inactive lipase.

The fact that Pekelharing [1912] ascribes to calcium the greatest part of the activating effect of the ash solution of the co-enzyme, without

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adducing, however, any figures to show that calcium is present in sufficient amount in this solution, induced me to estimate quantitatively the calcium contents of my extracts. Qualitative experiments showed that its quantity was very small and 200 cc. of the extract were therefore taken for the estimation, which was made by Aron's method, as modified by R. von der Heide [1914]. 200 cc. gave 0·0436 g. calcium oxalate = 0·02 % $CaCl_2$.

A series of experiments was next carried out, in which increasing amounts of calcium chloride were added to 5 cc. of a 0·2 % suspension of inactive lipase. After bringing up to equal volume with water and adding 5 cc. of olive oil emulsion the mixtures were incubated for 17 hours. The action of calcium chloride was compared with that of the original co-enzyme and its ash solution. The usual controls were made.

Calcium chloride content of mixture	Degree of lipoclasis: Titrated value cc. N/10 KOH	Degree of lipoclasis: Corrected value cc. N/10 KOH
M/7000	2·5	0
M/1400	2·5	0
M/700	5·0	+2·5
M/70	4·5	+2·0
M/7	3·1	+0·6
M/3·5	2·0	−0·5
Inactive lipase alone	2·5	0
Inactive lipase + co-enzyme (1 cc.)	12·4	8·3
Inactive lipase + ash solution (1 cc.)	11·0	8·1

From these results we may conclude that calcium chloride, if present in the digestion mixture to the amount of M/7000 to M/1400 has no effect on the lipoclasis. As the calcium content of 1 cc. of the ash solution of the co-enzyme corresponds to a M/4116 solution, it seems justifiable to conclude that the action of the ash solution is not due to calcium. Even if it were present in much larger proportion (M/700 to M/70) its effect would still be far below that of the ash solution itself.

I have also examined in a similar way the action of primary calcium phosphate, $CaH_4(PO_4)_2$, as this salt might possibly be present in the solution of the ash. The following results were obtained.

$CaH_4(PO_4)_2$ solution added cc.	Titration value cc. N/10 KOH	$CaH_4(PO_4)_2$ contents of the mixture	Degree of lipoclasis: Titrated value cc. N/10 KOH	Degree of lipoclasis: Corrected value cc. N/10 KOH
0·1 M/100	trace	M/7000	2·8	0·6
1·0 ,,	0·4	M/700	4·0	1·4
2·0 ,,	0·8	M/350	7·6	4·6
1·0 M/20	1·3	M/140	7·1	3·6
1·5 ,,	1·9	M/95	5·7	1·6
2·0 ,,	2·5	M/70	4·9	0·2

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These experiments show that primary calcium phosphate has a certain amount of activating action, which must, however, be ascribed to its phosphoric acid ions if we consider the results of the preceding experiments. But even its maximum effect (M/350) accounts for only about 50 % of that of the ash solution of the co-enzyme.

Whilst therefore admitting the possibility that calcium salts may act in the way suggested by Pekelharing[1] (*i.e.* by precipitating a small amount of fatty acid as an insoluble soap), it seems from the above experiments that its rôle as an activator of inactive lipase is in any case an insignificant one.

The action of sodium chloride on inactive lipase.

As sodium chloride forms a constituent of the co-enzyme solution, I have made a few experiments with this salt. 5 cc. of a 0·2 % suspension of inactive lipase to which increasing amounts of sodium chloride solution had been added, were brought up to equal volume with water, mixed with 5 cc. of oil emulsion and incubated for 19 hours. The results are given in the following table.

NaCl concentration of the mixture	Degree of lipoclasis: Titrated value cc. N/10 KOH	Degree of lipoclasis: Corrected value cc. N/10 KOH
M/700	1·8	0·9
M/175	2·2	1·3
M/100	3·0	2·1
M/70	3·9	3·0
M/17·5	2·8	1·9
M/10	2·7	1·8
M/7	2·2	1·3
Inactive lipase alone	0·9	0
Inactive lipase + co-enzyme (1 c.)	9·5	7·0

It would seem from these experiments that the action of sodium chloride is not of any great significance. Its effect increases to a certain limit, after which it decreases again. It may be mentioned here, however, that Dr Shaw-Mackenzie and the late Mr Myers-Ward occasionally observed a considerable activating influence of sodium chloride on inactive lipase. No explanation has as yet been found for these contradictory results.

Discussion.

In considering the state of our present knowledge of the conditions governing the activities of pancreatic lipase, it is essential to differentiate sharply between the lipase of glycerol extracts of pancreas and the lipase as

[1] See, however, Rona and Bien [1914], whose observations on the action of calcium on pancreatic and serum lipase do not favour this explanation.

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contained in pancreatic juice. The activity of the latter is in no way impaired by ordinary filtration and it is evident that the enzyme exists in a soluble condition in pancreatic juice. In glycerol extracts of pancreas, however, the material to which its lipoclastic activity is due occurs in a colloidal suspended condition and it requires a soluble co-enzyme for its activation.

The changes by means of which the insoluble lipase of the organ is transformed into the soluble enzyme of the pancreatic juice during the process of the secretory activity of the organ, are at present still unknown. With the methods at our disposal at present, it has also been found impossible to demonstrate the presence of a co-enzyme for the soluble lipase of pancreatic juice. It is, however, to be expected that the study of substances which possess a solvent action on the inactive lipase of glycerol extracts may give us a clue as to the conditions which govern the solubility of the lipase of pancreatic juice.

Various substances are now known which are able either to activate inactive lipase or to accelerate the action of active lipase. It seems that these substances may be divided into three groups: (1) The salts of Na, K, Ca, Ba, Mg; (2) organic and inorganic phosphates; and (3) bile salts and their derivatives. Very little is known, however, with regard to their mode of action.

In the case of the inactive lipase of pancreatic extracts any explanation is complicated by the fact that we are dealing with an insoluble enzyme acting on an equally insoluble zymolyte (fat). The action of neutral salts may therefore be partly due to a physical effect either on the degree of emulsification of the fat or on the degree of colloidal suspension of the enzyme. Pekelharing has further made it probable that the relatively small effect of electrolytes may be ascribed to the removal of one of the products of reaction (fatty acid) from the system.

This explanation seems, however, hardly admissible for the substances belonging to the second group. Their action may be due to a direct action on the enzyme or perhaps in the case of phosphates to the fact that their hydrogen ion concentration corresponds to the optimum activity of the enzyme. Another explanation of the action of phosphates may be their possible rôle in linking the inactive enzyme to the fat and giving rise to the intermediate formation of glycerophosphoric acid.

In the last class of substances, the bile salts, it has been made most probable by Rosenheim's observations that their active constituent, cholalic acid, acts directly on the enzyme. A similar conclusion has also been reached by Terroine [1910].

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The nature of the activating substances contained in a boiled watery extract of pancreas is not yet completely cleared up. Bile salts are absent and of the inorganic substances it seems that the main rôle is due to phosphates. The nature of the organic activator remains still to be discovered.

The expenses of this research have been defrayed out of a grant made to Dr Rosenheim from the Government Grant Committee of the Royal Society.

Conclusions.

(1) Phosphates represent the most active inorganic constituents of the co-enzyme of inactive lipase obtained from pancreatic extracts.

(2) Organic substances, probably organic phosphates, are also concerned in the activation of inactive lipase.

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VI. ON THE AUXO-LIPASE OF SERUM.

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The addition of small amounts of serum to pancreatic extracts increases their fat-splitting power to a remarkable degree. This fact was established by Pottevin [1903] and independently by J. A. Shaw-Mackenzie in his joint work with O. Rosenheim on the action of lipase contained in glycerol extracts of pancreas on olive oil and ethyl butyrate [1910]. The lipase of pancreatic juice (dog) as well as that of human pancreatic juice [Shaw-Mackenzie, 1912] are similarly influenced by serum. Minami [1912], apparently without knowledge of the previous work, recorded similar observations, using monobutyrin as the zymolyte.

With regard to the chemical nature of this accelerating substance, for which I propose the name of auxo-lipase[1], Rosenheim and Shaw-Mackenzie only made some preliminary statements, according to which it seemed to withstand the temperature of boiling water and was not destroyed by putrefaction. Further, it appeared to be soluble in dilute alcohol and to dialyse through membranes[2].

In a recent paper J. Mellanby and Woolley [1914] made a useful suggestion with regard to the nature of this reaction, which lends itself to experimental investigation. They consider that the increase of lipoclastic power on the addition of serum to pancreatic extracts is due to the protection of the lipase from the action of trypsin by the antitrypsin contained in the added serum.

The properties of auxo-lipase, as far as they have been investigated, suggest a connection with the co-enzyme of pancreatic lipase. The purpose of this investigation was in the first instance to compare the behaviour of

[1] Similar auxo-substances in serum have already been described in literature (see M. Falk, [1914]; Guggenheimer, Minami).

[2] I am informed that in later experiments Rosenheim and Shaw-Mackenzie were unable to confirm this preliminary statement.

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these two substances and to establish their identity or difference. Further, the relationship of auxo-lipase to the antitrypsin of serum was experimentally investigated. This relationship is of interest since it has been shown by Shaw-Mackenzie [1912, 1913] that variations in amount of the auxo-lipase of serum together with variations in amount of antitrypsin may be of diagnostic value in certain pathological conditions.

Experimental.

Since the action of the auxo-lipase is not specific, I have limited my work, for convenience sake, to the investigation of ox-serum. Olive oil was chosen as the zymolyte, as this fat gives much larger values than those obtained by the action of pancreatic lipase on various esters of the lower fatty acids. The method used for the estimation of the lipoclastic activity was the same as that of Rosenheim and Shaw-Mackenzie. The precautions necessary for comparable results have been fully described in the communication of Umeda [1915]. Instead of phenolphthalein, I used, however, a 2 % alcoholic solution of thymolphthalein. This indicator, introduced by Sörensen [1908], changes into a deep blue with alkali, a change which appears to afford a sharper end point in the presence of the pigments of the serum than that of phenolphthalein. All the experiments were carried out in duplicate.

The presence of auxo-lipase in serum.

In order to convince myself of the existence of auxo-lipase in serum, I carried out a large number of preliminary experiments with sera and pancreatic extracts obtained at different times. The reaction was found to be remarkably constant, and the following experiment, chosen at random from many, illustrates the result obtained.

A pancreatic extract was prepared by mixing 2·5 kg. of finely minced pig's pancreas with 5 litres of glycerol. The mixture was strained through two layers of muslin after 24 hours. Before use one part of this extract was diluted with two parts of water. 1 cc. of this diluted extract, which will be designated as "lipase" in the following, was used in each experiment, the serum being added next and the fat emulsion last. The mixture was incubated for 17–24 hours, this period being chosen for convenience sake. In the following table the effect due to auxo-lipase is shown in the last column, expressed in cc. N/10 KOH[1].

[1] The amounts of N/10 KOH required by the pancreatic extract and by the emulsion alone have in all cases been deducted from the results given in this and the following tables.

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Lipase cc.	Serum cc.	Emulsion cc.	Water cc.	N/10 KOH cc.	Effect of Auxo-lipase
1	–	3	3	13·3	—
1	1	3	2	27·1	13·8
1	2	3	1	36·7	23·4
1	3	3	–	37·3	24·0

When using the same amounts of serum and pancreatic extracts of different origin and incubating for the same time practically identical figures were obtained, the variations not exceeding ±1 cc. N/10 KOH.

In the following series of experiments a smaller amount of pancreatic extract was taken and increasing amounts of serum from 0·2 cc. to 3·0 cc. were added. The results are shown in the following table.

Lipase cc.	Serum cc.	Emulsion cc.	Water cc.	N/10 KOH cc.	Effect of Auxo-lipase
0·5	—	3	3·5	5·9	—
0·5	0·2	3	3·3	17·7	11·8
0·5	0·4	3	3·1	20·8	14·9
0·5	0·6	3	2·9	22·4	16·5
0·5	0·8	3	2·7	24·9	19·0
0·5	1·0	3	2·5	28·2	22·3
0·5	1·5	3	2·0	31·9	26·0
0·5	2·0	3	1·5	33·2	27·3
0·5	3·0	3	0·5	35·8	29·9

Fig. 1 (p. 56) gives a graphic representation of the increase of the auxo-lipoclastic effect produced on the addition of increasing amounts of serum.

The auxo-lipase of serum is not only able to accelerate the action of active lipase but also to activate inactive lipase. The few observations which I have made in confirmation of Rosenheim's [1910] statements, were carried out with a sample of inactive lipase prepared by Dr Umeda in this laboratory. 10 mg. of inactive lipase suspended in 1 cc. of water, by itself practically without any action on olive oil, produced together with 1 cc. of co-enzyme an effect nearly equal to that of 1 cc. of my own diluted pancreatic extract. The following table shows the results produced on the addition of 2 cc. of serum to 10 mg. of inactive lipase.

Inactive Lipase 10 mg. in 1 cc.	Serum I	Serum II	Serum III	Co-enzyme	Lipoclastic effect cc. N/10 KOH
1	–	–	–	–	0·3
1	–	–	–	1	10·7
1	2	–	–	–	28·1
1	–	2	–	–	27·4
1	–	–	2	–	28·7

Not only has the addition of serum activated inactive lipase, but the effect produced is even greater than that of the co-enzyme.

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These experiments leave no doubt about the existence of the auxo-lipoclastic action of serum. It may be mentioned, however, that in order to observe this reaction, it is necessary that enzyme, serum, and zymolyte shall be mixed in the order given. The following experiment illustrates the effect produced by an alteration in the order of mixing. In experiment (*a*) lipase and serum were mixed before the addition of the fat emulsion, whilst in

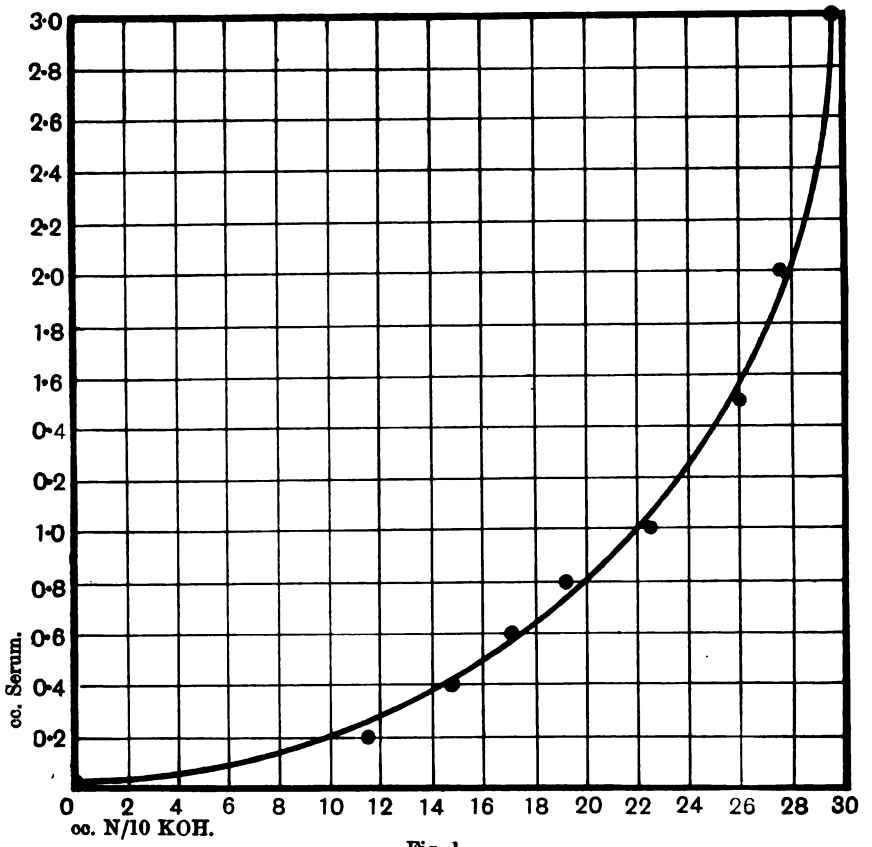

Fig. 1.

experiment (*b*) serum was added to the mixture of lipase and fat emulsion. The mixtures were brought to an equal volume by the addition of water and incubated for 24 hours.

(*a*)				(*b*)			
Lipase cc.	Serum cc.	Emulsion cc.	Effect of Auxo-lipase	Lipase cc.	Emulsion cc.	Serum cc.	Effect of Auxo-lipase
1	1	3	14·6	1	3	1	6·7
1	2	3	26·1	1	3	2	10·2
1	3	3	27·8	1	3	3	12·7

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This experiment clearly demonstrates that by altering the order of mixing, the action of the auxo-lipase is diminished by over 50 %. The significance of this fact for the explanation of the reaction will be discussed later.

The stability of auxo-lipase.

In order to test the stability of the auxo-lipoclastic substance contained in serum, its effect was estimated in fresh serum and in portions which had been kept for over a month with and without toluene. The serum kept without a preservative had become putrid.

No difference was found between the action of fresh serum and that kept under toluene. To save repetition the figures are not given. The auxo-lipase in putrefied serum, on the other hand, had not only not been destroyed, but even slightly increased. In this and the following experiments 1 cc. of lipase and 3 cc. of emulsion were used and the mixtures brought up to equal volume by the addition of water. The necessary corrections were made as before. The figures were:

Putrefied serum cc.	Effect of Auxo-lipase
1	16·0
2	23·4
3	27·2

This increase may be due to concentration of the serum owing to evaporation, as it had been kept in an open flask in order to encourage putrefaction. These experiments confirm the results previously obtained in this laboratory that serum retains its auxo-lipoclastic activity even when kept for many months.

The heat-resistance of auxo-lipase.

This was tested by keeping undiluted serum for 0·5 hour in a water-bath at a temperature of 60°, and another portion, which had been diluted with an equal volume of water, for 10 minutes in a boiling water-bath. The various sera were tested in the usual way. The results are given in the following table, showing the effect of the auxo-lipase contained in 1, 2 and 3 cc. of serum, expressed in cc. N/10 KOH.

Fresh serum	Serum kept at 60° for 0·5 hour	Diluted serum	Diluted serum kept at 100° for 10 minutes
13·8	14·2	4·6	9·1
23·4	25·9	8·3	24·8
24·0	28·0	18·4	29·5

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Auxo-lipase is therefore not destroyed by heating to 60° or 100°, but its action is even increased considerably. The significance of this fact and its bearing on the relationship of antitrypsin and auxo-lipase will be discussed later.

The effect of the removal of the proteins.

It was hoped to obtain more information about the nature of the auxo-substance by the removal of the proteins, which was effected (*a*) by heat coagulation in the presence of acetic acid, (*b*) by alcohol, and (*c*) by means of colloidal iron. In each case, however, the active substance was either carried down together with the protein or rendered inactive, and further the protein-free filtrates exerted even an inhibitory action on pancreatic lipase.

(*a*) *Heat coagulation in presence of acetic acid.* It was found that the presence of acetic acid did not interfere with the reaction at 37°, but that when the serum had been heated to 100° after the addition of acetic acid, its accelerating action was converted into an inhibition of the lipase.

A few drops of glacial acetic acid were added to 25 cc. serum, until an acid reaction to litmus was distinct.

Quantities of 2 cc. and 3 cc. were measured into a series of flasks. One series was kept for 10 minutes at 100° in a water-bath. The action of heated and unheated acidified serum was compared with that of normal serum in the usual way. The figures represent the increase or decrease (expressed in cc. N/10 KOH) of the lipoclastic action of 1 cc. of lipase after the addition of 2 and 3 cc. of the sera.

Normal serum	Serum + acetic acid (unheated)	Serum + acetic acid (heated for 10 minutes at 100°)
+16·4	+17·8	−5·5
+21·1	+21·2	−6·0

It seemed to be interesting to analyse the inhibitory action of the heated acidified serum further and to investigate whether the auxo-lipase was actually destroyed or if it was possible to restore its action by subsequent neutralisation.

25 cc. of serum were diluted with an equal volume of water, acidified with a few drops of acetic acid and kept at 100° for 10 minutes. The coagulated protein was filtered, finely ground in a mortar, boiled up with distilled water and again filtered and washed. It was then suspended in 50 cc. of water and one half of it neutralised with N/10 KOH.

The combined filtrates were concentrated, made up to 25 cc. with water and one half of the solution was neutralised with N/10 KOH.

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The following results were obtained when testing the action of 2 cc. and 3 cc. of these fluids on lipase as before.

Protein suspension		Filtrate	
Washed	Neutralised	Acid	Neutralised
−6·4	+ 7·0	−6·0	−1·2
−5·7	+10·8	−5·8	−0·9

It will be seen that both the coagulated protein and the filtrate acquire an inhibitory action, when serum is heated in the presence of acetic acid. It seems further that the auxo-lipase is carried down with the coagulated protein, and is set free again by careful neutralisation, whilst the neutralised filtrate still remains inhibitory.

It may be mentioned in this connection that I have found in some preliminary experiments that milk also contains a substance which accelerates lipoclastic action. In this case also it is carried down by acetic acid with the caseinogen of milk, and set free again by the addition of a small amount of alkali.

(*b*) *Removal of proteins by alcohol.* 40 cc. of serum were poured into 400 cc. of boiling absolute alcohol, kept on a water-bath for half an hour and filtered. The coagulated protein was freed from alcohol by drying at 40°, finely powdered and divided into four parts. One part was suspended in 20 cc. of water. Another part was suspended and boiled with 20 cc. of water and finally to another part, suspended in 20 cc. of water, a few drops of N/10 KOH were added so as to make its reaction faintly alkaline to litmus.

The alcoholic filtrate was evaporated to dryness on the water-bath and the residue taken up with 200 cc. of absolute alcohol. After filtration the alcohol was evaporated and the residue taken up with 40 cc. of water.

The action of the various fractions on lipase was tested as before, 1 cc. and 2 cc. of each fraction being used. The results are given below.

Alcohol-Coagulum			Alcohol Extract
Suspended	Boiled	Neutralised	
+1·0	+3·4	+ 7·2	−0·1
+4·7	+7·6	+20·8	+0·7

Here again we see that the auxo-lipase is carried down with the coagulated protein, but in distinction from the acetic acid coagulum it retains its activity. Further, in analogy with its behaviour in the original serum, its activity is increased by boiling and still further by the addition of a small quantity of

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alkali. The protein-free alcoholic extract is practically inactive. The insolubility of auxo-lipase in absolute alcohol differentiates this substance from the co-enzyme of lipase contained in watery pancreatic extracts.

(c) *Removal of proteins by colloidal ferric hydroxide.* By the use of this method [Michaelis and Rona, 1908] every trace of protein is removed from serum in the cold, without the introduction of any foreign substance into the filtrate. To 25 cc. of serum, diluted with 250 cc. of water, 20 cc. of colloidal ferric hydroxide solution were gradually added. The precipitated colloids were filtered off and washed until the wash-water was free from chlorides. The clear filtrate and wash-water were evaporated to 25 cc. The solution, containing the electrolytes and crystalloids of serum, was found to possess an inhibitory action on lipase.

When tested in the usual way the inhibitory action (expressed in cc. N/10 KOH) was: for 1 cc., — 0·5, and for 2 cc., — 1·1.

Evidently the auxo-lipase is removed together with the proteins by colloidal iron[1], a fact which speaks in favour of the colloidal nature of this substance. In order to test this still further, the behaviour of the auxo-lipase on dialysis was investigated.

The behaviour of auxo-lipase on dialysis.

As all the experiments which I carried out in this direction gave identical results, it will be sufficient to describe one of them in full. 30 cc. of serum were dialysed for seven days in a parchment diffusion shell (Schleicher and Schüll), toluene being used as a preservative. The distilled water was changed twice daily until it was free from chlorides. The dialysate was concentrated to 30 cc.

The dialysed fluid, which measured 48 cc., was divided into two parts. One half was used as such, whilst the other half was centrifugalised in order to test the action of the euglobulin fraction separately. Further, one part of the centrifugalised fluid, containing the serum albumin and pseudoglobulin, was heated on a water-bath for 10 minutes. The various fractions were tested as before, 1 cc., 2 cc. and 3 cc. being used of each and allowance being made for the dilution of the serum during dialysis. The results are given in the following table, the increase or decrease of the lipoclastic action being expressed in cc. N/10 KOH.

[1] The colloidal precipitate was not tested, as the large amount of iron in it was expected to render the result inconclusive.

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Original serum	Dialysed serum	Dialysate	Albumin + Pseudoglobulin Unheated	Albumin + Pseudoglobulin Heated	Euglobulin
+13·8	+16·4	−3·2	+18·4	+21·1	+2·0
+23·4	+21·3	−3·8	+21·4	+28·3	+4·2
+24·0	+24·1	−6·5	+24·4	+32·2	+4·3

The colloidal nature of the auxo-lipase is strikingly confirmed by this experiment, the action of the dialysed serum being little different from that of the original serum. Here again we meet with a characteristic difference between the behaviour of auxo-lipase and the co-enzyme of lipase, which diffuses easily [Umeda, 1915]. The experiments on fractionation of the serum proteins, as far as they go, point to an association of the auxo-lipase with the albumin and pseudoglobulin fraction. I was unable, owing to lack of time, to complete the fractionation.

The inhibitory action exerted by the dialysate suggests the question whether the inhibition is due to a substance of organic nature, or whether the electrolytes are responsible for it. As it has been shown by Umeda [1915] that the inorganic constituents of the co-enzyme of lipase are largely concerned in the activation of inactive lipase, an investigation of this question was likely to throw further light on the question of the identity of auxo-lipase and the co-enzyme.

The action of the mineral substances of serum.

The mineral substances remaining after the incineration of the whole serum as well as those of the dialysate were examined. 10 cc. of serum and 10 cc. of the dialysate (see above) were incinerated, the usual precautions being taken to avoid loss. The ash was in each case taken up with water and neutralised with N/10 HCl, using methyl-red as an indicator. The solution was evaporated to dryness, in order to drive off any excess of hydrochloric acid and the residue made up to 10 cc. with water. The following table shows the results, expressed in cc. N/10 KOH. For comparison the results obtained from the dialysate before incineration are added.

	1 cc.	2 cc.	3 cc.
Dialysate	−3·2	−3·8	−6·5
Ash of dialysate	−1·1	−1·7	−4·8
Ash of serum	−2·5	−2·9	−4·2

From these results it would seem that a large part of the inhibitory action of the dialysate is due to its mineral constituents, but that a certain amount of it is of an organic nature and lost by combustion. In sharp distinction

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from the inorganic constituents of the co-enzyme of lipase, those of the serum not only fail to produce an accelerating effect but even inhibit lipoclastic activity, thus furnishing further evidence against the identity of auxo-lipase and co-enzyme.

The effect of serum lipoids.

In view of the fact that auxo-lipase is non-dialysable the possibility of its lipoid nature had to be considered. It has already been shown above, that an alcoholic extract of serum is practically inactive. I have further investigated the solubility of auxo-lipase in ether. For this purpose I made use of a serum powder obtained by drying serum in Petri dishes at 40° by means of an electric fan. I have convinced myself that a solution of this powder possesses the same effect as the original serum. 2 g. serum powder, corresponding to 20 cc. serum, were extracted in a Soxhlet apparatus with ether for four days. The residue of the ether extract was suspended in 20 cc. of water and tested in the usual way. Its effect on lipase (expressed in cc. N/10 KOH) was: 1 cc., 0; 2 cc., − 1·8; 3 cc. − 2·6.

This result together with the negative result produced by the alcohol extract shows that auxo-lipase is not of lipoid nature.

The relationship of auxo-lipase to antitrypsin.

It has already been mentioned above that the well-known presence of antitrypsin in serum has been suggested as affording an explanation of the auxo-lipoclastic action of serum. According to this view antitrypsin by inhibiting the action of trypsin prevents also the supposed destructive action of this enzyme on lipase.

The above described experiments on the heat resistance of auxo-lipase (p. 57) make it impossible to accept this view. According to Cathcart [1904] antitrypsin of serum—the albumin fraction—is destroyed on boiling and "is rapidly injured by heating. A temperature of 55° for half an hour suffices both in the presence and absence of alkali to destroy to a large extent the anti-action." Further, Hedin [1906] showed that it is readily destroyed at blood-heat by dilute acetic acid. In the above described experiments serum was kept at 60° for 0·5 hour and at 100° for 10 minutes, under which conditions antitrypsin is undoubtedly injured and destroyed. In spite of this the effect of the auxo-lipase was found in both cases considerably increased. Further, the action of auxo-lipase was not affected by dilute acetic acid at blood temperature.

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Further experimental evidence against the assumed interaction of trypsin, antitrypsin and lipase is furnished by the following observation. Diluted pancreatic extract (1 : 3) was incubated for 24 hours, toluene having been added. The solution had acquired a powerful tryptic action which was found on comparison to be equal to that of a 1 % solution of Kahlbaum's trypsin. Under these conditions the lipase of pancreatic juice is entirely destroyed. It was therefore to be expected, if the lipase of pancreatic extract behaved like that of pancreatic juice, that the subsequent addition of serum to incubated pancreatic extract would fail to produce any effect, if its action were merely due to the antitrypsin contained in it. The following results were obtained on comparing the activity of freshly diluted and of incubated pancreatic extract. The effect due to auxo-lipase is expressed in cc. N/10 KOH.

Pancreatic extract				
Freshly diluted	Incubated for 24 h.	Serum	N/10 KOH cc.	Auxo-lipase
1	–	–	9·6	—
–	1	–	10·3	—
–	1	1	29·2	18·9
–	1	2	34·8	24·5

It will be seen that in distinction from the lipase of pancreatic juice, that of pancreatic extract is in no way affected by incubation for 24 hours, in spite of the development of trypsin. This protection may possibly be due to the presence of glycerol. Further, on addition of serum the action of auxo-lipase is even somewhat more strongly marked than in the case of freshly diluted extract (see p. 55).

Discussion.

The experimental material now available enables us to define the general properties of auxo-lipase somewhat more closely and to compare them with those of the co-enzyme of lipase.

Auxo-lipase of serum is thermostable, non-dialysable, insoluble in absolute alcohol and ether, and destroyed by incineration. It is associated with the serum-proteins and carried down with them by alcohol, by heat coagulation or by colloidal ferric hydroxide. Of the serum proteins the albumin and pseudoglobulin fraction appear to have a greater affinity to auxo-lipase than the euglobulin fraction. It is also noteworthy that serum contains an organic anti-lipoclastic substance the action of which is, however, masked by that of the auxo-lipase.

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From its inability to pass through a dialysing membrane, one may conclude that auxo-lipase is of a colloidal nature, a conclusion which is strengthened by its behaviour towards colloidal ferric hydroxide.

This property sharply differentiates it from the co-enzyme of lipase, which dialyses easily and the activity of which, moreover, is not greatly diminished by incineration. The experimental evidence seems therefore to justify the conclusion that the auxo-lipase of serum and the co-enzyme of lipase are not identical.

With regard to the relationship of auxo-lipase and antitrypsin my experiments make it unfortunately impossible to accept the very plausible explanation advanced by Mellanby and Woolley. It may further be pointed out that from the experimental work of Shaw-Mackenzie [1912, 1913] it follows clearly that the antitryptic index of serum does not run parallel to its content in auxo-lipase and that, for instance, in the same individual a high antitryptic index may be associated with a low auxo-lipase content or a high auxo-lipase content of the serum associated with a low antitryptic index.

In dealing with this complex reaction the important factor of the physical condition of enzyme and zymolyte must not be lost sight of. As both these are insoluble, the presence of a colloidal solution like serum may influence the degree of emulsification of the zymolyte (fat) or the colloidal suspension of the enzyme (lipase).

From the experiment recorded above, however, in which the importance of the order of mixing was emphasised, we may conclude that this reaction is due to a direct action of the auxo-lipase of serum on the enzyme.

The expenses of this research have been defrayed out of a grant made to Dr Rosenheim from the Government Grant Committee of the Royal Society.

Conclusions.

1. The auxo-lipase of ox-serum is non-dialysable, thermostable and destroyed by incineration.
2. The auxo-lipase of serum and the co-enzyme of lipase are not identical.
3. No relationship could be demonstrated between the antitrypsin and the auxo-lipase of serum.

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VII. A SIMPLE HYDROGEN ELECTRODE.

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For methods to estimate the true reaction of a liquid biologists are indebted to a series of investigators, of whom only Sörensen [1909], Hasselbalch [1911, 1913], Michaelis [1909, 1912] and Walpole [1913] may be mentioned. The last of these has succeeded in constructing hydrogen electrodes, which allow very rapid and easy estimations.

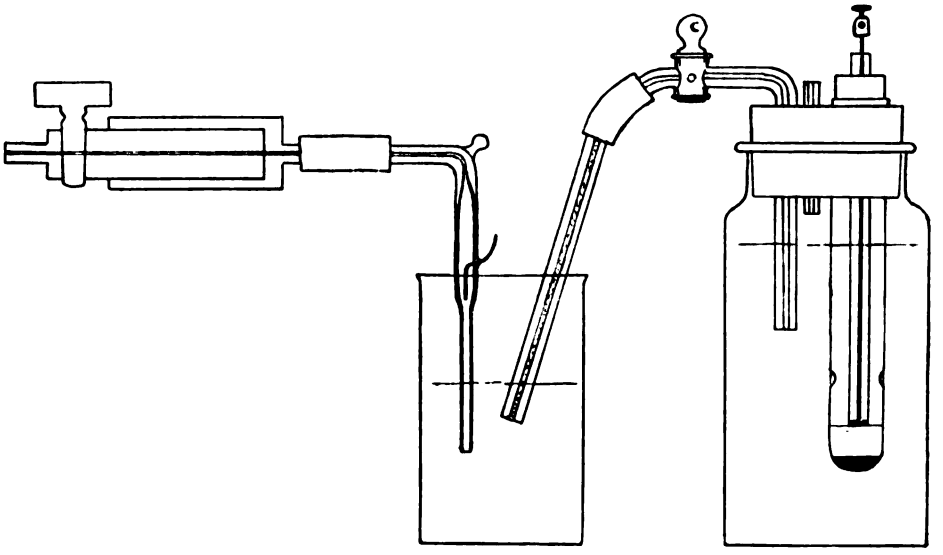

Fig. 1.

However, the apparatus, which Walpole used, is not simple and strong enough for continuous use and does not give all the security which is obtainable.

In the accompanying Figure 1 a modified arrangement is represented, which has proved still more serviceable for everyday work on the open bench without a constant temperature bath.

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The hydrogen electrode is here simply a bent piece of glass tube (Jena glass), with a platinum wire sealed in its wall. The form of this glass tube, as will be shown further on, is carefully devised to meet all the requirements brought out by previous investigations. By means of a piece of rubber tubing it is connected to a perforated brass or copper syringe.

In carrying out an estimation, the hydrogen is first admitted through the tap, while the piston is still pushed home. After shutting the tap, the gas is nearly all contained in the electrode tube itself. By drawing the piston out and in several times, equilibrium between the enclosed hydrogen and the

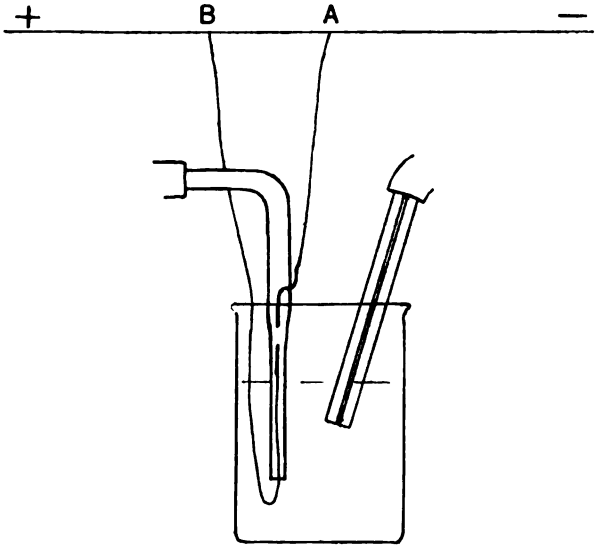

Fig. 2.

carbonic acid, which the liquid may contain, is better secured with this arrangement than with the apparatus of Walpole, where there is a considerable dead space behind the electrode tube. The sample, which is at last drawn up into the tube, when the gas-equilibrium is established, is evidently a fresh one.

Michaelis found, that a definite potential was much sooner attained, if the electrode only just dipped into the liquid. Konikoff [1913] drew attention to the fact, that dissolved oxygen might combine with the hydrogen of the electrode, which explained the fact observed by Michaelis. By submerging only a small point of the electrode a lasting saturation with hydrogen is sooner and better secured.

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As the liquids to be examined in a biological laboratory often contain carbonic acid and oxygen, dissolved or loosely bound, it is important to take account of both of them.

As long as the liquid near the electrode contains some oxygen, this combines readily under the influence of the platinum black with the hydrogen of the electrode, thereby lowering its solution tension, i.e. its potential.

To secure a quick and automatic removal of the oxygen from the actual sample in the electrode tube I first tried to bring into this tube a second piece of blacked platinum wire, not connected with the electrode.

Figure 2 illustrates the experiments, made with this arrangement. Only the hydrogen electrode and the stretched wire *AB*, which served as potentiometer, are represented here. *A* is the point where the slide, connected with the gas cell, compensates the current from the accumulator. If now, before repeating the experiment with the same liquid, we bring inside the electrometer tube a platinum or gold wire with some platinum black on it, the other end of the wire coming out free into the air, the electrometer shows a large current, if the slide is pressed down in point *A*. Generally the slide has to be moved a considerable distance to the left (positive side), seldom to the right. If, however, we press the free end of the second wire, insulating it from the fingers with a piece of rubber tubing, on the potentiometer wire to the left of *A*, a point *B* can be found, at which the influence of the second wire is eliminated. This equilibrium does not last long. To maintain it we must gradually move the point *B* further to the left.

The interpretation of these facts is as follows. Ionised hydrogen can pass freely from the one blacked wire to the other. The hydrogen in the platinum black of the second wire will soon be oxidised by the oxygen, dissolved in the liquid, to which it is on all sides exposed; its solution tension will be lowered and it will therefore take more and more hydrogen out of the electrode. This transport of hydrogen is stopped, if we bring the second wire to a potential so much higher than that of the electrode, that the difference in solution tension of the hydrogen in the two wires is just compensated. The progressive oxidation of the hydrogen in the second wire will require a gradually increasing potential difference to do this.

If we pass hydrogen again through the electrode tube, while the second wire is inside it, both blacked wires will be saturated with hydrogen and therefore show no potential difference. This was observed repeatedly. Shortly after a renewed passage of hydrogen the point *B* came very near to *A* and then had gradually to be moved again to the left.

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After saturating the liquid in the beaker (Sörensen's phosphate mixtures were mostly used in these experiments) with oxygen, *B* must be moved still more quickly away from *A*.

A blank platinum wire, introduced into the electrode tube, gives no effect at all, if its other end comes out free into the air. Neither does a gold or copper wire. If the other end is pressed on the potentiometer wire, very irregular effects are obtained by polarisation.

By these experiments it became clear, that the necessary removal of the oxygen from the liquid near the electrode wire had to be performed by the blacked platinum electrode itself.

I therefore placed the electrode as indicated in Fig. 1 with its point near the narrow part of the tube. If now, after securing the carbonic acid equilibrium as described in the beginning, the surface of the actual sample of liquid to be examined is several times drawn up and down the blacked wire (not higher and not lower), it is quickly reduced by the hydrogen in the relatively large surface of blacked platinum wire. The diameter of the platinum wire is about 0·6 mm., the inside diameter of the tube around it about 4 mm. By then pushing the reduced liquid down the long narrow end of the electrode tube until its surface is near the platinum point, we prevent original, not reduced, liquid from diffusing into the neighbourhood of the electrode.

With this arrangement it did not prove absolutely necessary to make the liquid just touch the electrode, though it is quite as simple and safer to observe this precaution.

These operations for securing the equilibrium of the carbonic acid and the removal of the oxygen take only a very short time, less than a minute.

Before this improvement was worked out, an increase of a few millivolts was generally noticed in the potential during the first minutes. The fact, that freshly boiled liquids did not show this phenomenon, drew attention to the marked influence, above described, of oxygen, often even of some dissolved air.

The glass bead on the electrode tube serves to fasten the thin copper wire, which is soldered to the outer end of the platinum wire.

The other half of the galvanic cell, represented in Fig. 1, has also proved to be very useful. A bottle of some 150 cc., filled with saturated KCl, is closed by a rubber stopper, through which three tubes pass. The wider one is the mercury-calomel-saturated KCl electrode proper, a tube, closed at the bottom, but with two openings in its side. The second is a capillary tube

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with a tap and connected by a small piece of rubber tubing to a capillary, filled with cotton wool. The third tube serves simply to prevent air-pressure in the top of the bottle from causing inconvenience. This electrode bottle has been in use for months without needing any refilling or cleaning. The tap, which is not greased, remains always closed. There proved to be sufficient conductivity and even some capillary transport of KCl solution down the open cotton wool tube, purifying automatically the open end of it, which has dipped in the examined liquid; usually, in the morning, some KCl was found to have precipitated round the opening. Both glass tubes, dipping in the titrated liquid, may of course have smaller dimensions if the available volume is very small. The usual capacity of my electrode tube was about 0·3 cc.

Summary.

A simple arrangement for the estimation of the true reaction of liquids, which remains always ready for use, is described and explained. It is in some points an improvement on Walpole's electrometric titrating apparatus for the open bench, special provision being made for allowing instantaneous and accurate estimations even in liquids, which contain carbonic acid and oxygen.

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VIII. ON THE CAUSE AND SIGNIFICANCE OF AN ABNORMAL REACTION OBTAINED IN TESTING URINE FOR SUGAR WITH FEHLING'S SOLUTION.

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(*Received February 3rd, 1915.*)

The investigations recorded here were carried out as the result of observations made on a urine obtained accidentally from the Infirmary. The urine was supposed to contain glucose and did, in fact, give the usual tests for glucose. Nylander's test was positive; with phenylhydrazine typical glucosazone crystals were obtained; it fermented and it showed a dextrorotation indicating a percentage of 1·5 % sugar calculated as glucose. But when Fehling's test was applied, a curious and striking abnormality was observed. On following the usual routine method of heating to boiling point, in two separate test tubes, equal quantities of urine and of half diluted Fehling's solution and pouring the two hot solutions together a sudden change took place at once: a deep scarlet-red transparent solution was obtained. If this solution was observed in transmitted light without further heating, it began to darken after about ten seconds, at first slightly, then more rapidly. At the same time the solution became less and less transparent and eventually after about a minute a very fine black precipitate appeared, which remained suspended in the fluid. The precipitate was so fine that on filtering it passed partly through the filter paper. If the mixture was allowed to stand for 24 hours, the precipitate became partly aggregated in soft slimy masses, which settled out at the bottom of the vessel. It could then be removed by filtration.

In studying the conditions under which the reaction took place it was noted that it was possible to prevent this abnormal reaction from taking place by simply diluting the urine with an equal volume of water. If Fehling's test was applied to such a diluted urine the typical result—a yellow or red precipitate—was obtained.

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If Fehling's solution and the undiluted urine were mixed in the cold and then heated together to boiling point (instead of heating them separately and mixing them when hot) a red precipitate was formed at first, which on further heating gradually darkened. This darkening became less and less marked when the relative amount of Fehling's solution with which a given volume of urine was mixed, was increased. The opposite condition—using an excess of urine and relatively little Fehling's solution—produced again a darkening of the cuprous oxide which was formed at first. If the red cuprous oxide obtained by heating together an excess of Fehling's solution and relatively little urine was added to a further quantity of the urine, and the mixture heated, the red precipitate again showed a darkening. It is perhaps necessary to state that the darkening of the fluid is quite different from that due to the action of alkali on sugar and that it is not due to the caramelisation of the sugar.

On investigating the nature of the black precipitate, it was thought at first from its physical appearance to be of an organic nature, perhaps a combination of copper with an abnormal organic constituent of urine. It was found, however, that the precipitate was not of an organic nature, for on heating weighed quantities of the precipitate in a crucible, it showed an increase in weight and formed a black mass, different in appearance from the original black precipitate and identical in appearance with cupric oxide, which, in fact, it was, as it yielded copper by being heated in a current of hydrogen. On treating the original black precipitate with acids, it was found that it dissolved readily in nitric acid with the evolution of brown fumes and formed a clear green solution: it was not readily affected by hydrochloric acid or sulphuric acid. It behaves therefore in the same way as metallic copper and is, in fact, copper in an extremely fine state of division, finer even than the copper obtained by reducing a cupric salt by means of a watery solution of glucose as described below. If the filter paper which has been used for the filtration of the precipitate is allowed to dry in the air, it acquires the characteristic green colour of basic copper carbonate (verdigris).

The fluid from which the precipitate had separated out had in transmitted light sometimes a brown, sometimes a greenish-black appearance, while in reflected light it was black, thus suggesting that it represented a suspension of very fine particles of the material which furnished the black precipitate, in other words that it was a colloidal solution of copper. This was confirmed by the fact that hydrogen peroxide was decomposed when added to the warm colloidal solution.

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The conclusion arrived at, that the black precipitate is finely divided metallic copper, is less surprising since I have found that Fehling's solution is readily reduced by glucose to metallic copper. It was thought, at first, that reduction to copper would take place only if cuprous oxide were present in colloidal solution, and that therefore the presence of such substances as creatinine, which are capable of holding cuprous oxide in solution, was necessary. But this is not the case.

If a hot concentrated solution of glucose, or solid glucose, is added in excess to boiling Fehling's solution, one notices as the first change the well-known formation of red or yellow cuprous oxide. If heating is now continued for one or two minutes, this red or yellow precipitate darkens and the whole mixture assumes a dark brown colour. If at that stage the flame is removed, so that boiling is interrupted, an evolution of gas will be seen to be proceeding. If boiling is continued for three minutes a reddish brown heavy precipitate is obtained which rapidly settles at the bottom of the vessel and which, when washed and dried in a steam bath, is finally obtained as a fine powder having the characteristic colour of copper. It dissolves in nitric acid with the evolution of brown fumes. This and the increase in weight obtained on heating show that the precipitate consists almost entirely of metallic copper admixed probably with a small amount of cuprous oxide, which has not been further reduced.

The increase of weight obtained on heating should be 25·1 per cent. if the precipitate were metallic copper and 11·2 per cent. if the precipitate were cuprous oxide. The following figures were obtained with three different preparations:

1.	0·4274 g.	precipitate gave	0·5202 g. CuO.	Increase in weight,		21·71 %.
2.	0·2479 g.	„ „	0·3056 g. CuO.	„	„	23·3 %.
3.	0·2385 g.	„ „	0·2954 g. CuO.	„	„	23·85 %.

The last preparation had been dried in a current of nitrogen, and heated in a current of oxygen. As a control the CuO obtained was reduced again by heating in a current of hydrogen.

0·2954 g. CuO gave 0·2362 g. Cu. Decrease in weight, 25·06 %.

The only difference which exists between the reduction produced by pure glucose solution and that brought about by this urine consists in the state of division. Since in the latter case one obtains at first a colloidal solution of cuprous oxide, which is then further reduced to a colloidal solution of copper, the black precipitate which one eventually obtains as the result of the "coagulation" of the colloidal copper solution represents a

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much finer state of division of the metal than the typically copper-coloured precipitate obtained with glucose in watery solution.

The black precipitate obtained from the urine was, of course, not chemically pure metallic copper, as it was mixed with the phosphates which had separated out from the urine as the result of boiling it with the alkaline Fehling's solution. It was probably also contaminated with some organic material adherent to it. Owing to this fact the increase in weight obtained on heating weighed quantities of the dried precipitate varied considerably (from 10 % to 14 %) and was below the value calculated for pure copper (25·1 %), but in some cases above that calculated for cuprous oxide (11·2 %).

It is now easy to understand the abnormal reaction described at the outset of this paper. When the heated urine and Fehling's solution were mixed, the glucose present in the urine at once reduced the cupric oxide to cuprous oxide. This, however, remained in solution for reasons which will be discussed presently, and then produced the transparent scarlet-red solution that has been described. This colloidal solution was then further reduced by the glucose to colloidal copper, which at first formed a colloidal solution from which it was gradually precipitated to form the black slimy masses of finely divided copper.

The question now arises: why is this reduction to metallic copper by urine containing glucose so rare, as it evidently is, since it has never been described? The answer is to be found in the following facts. The urine had the colour of normal urine. On diluting it with an equal volume of water, it gave, as has already been stated, the typical reduction with the appearance of cuprous oxide. On inquiry at the ward it was found that the total volume of urine excreted by the patient was rather below than above the normal. Dr R. A. Fleming, under whose care the patient was, and to whom I am greatly indebted for his readiness to place the material at my disposal, has very kindly given me all the details concerning the clinical aspects of the case, from which the following statement has been abstracted.

The case was demonstrated by Dr Fleming at a meeting of the Edinburgh Medico-Chirurgical Society [1914].

"The patient was a young woman, age 22 years, weight 8 st. 3¾ lbs. She complained of 'weakness and tiredness' from which she had suffered for three years. Six months before admission to the hospital her doctor discovered sugar in the urine, for which she was treated by being placed on a strict diabetic diet.

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The patient was very neurotic and frequently complained of 'Globus Hystericus' with palpitations on any excitement. She also had a degree of pharyngeal anaesthesia common in hysterical subjects. Apart from the excretion of sugar in the urine no other symptoms of disease were found.

The urine varied in amount from 24 oz. up to 52 oz., with an average of about 30 oz. in the 24 hours. The specific gravity of the urine varied from 1·030 to 1·035. There was no albumin in the urine. The amount of sugar varied greatly, from 0·4 grains per oz. and about 15 grains per day, to 5 grains per oz. and about 150 grains per day. Dietetic measures did not markedly affect the amount of sugar excreted. Apart from these measures she was treated also at first with codeine, later with a tonic containing strychnine. It was noted that whenever the sugar diminished the feeling of 'tiredness' was less marked.

After three months in hospital the patient gained 8 lbs. in weight and went out, feeling much better, although the excretion of sugar persisted."

Briefly summarised, the case is not a typical case of diabetes mellitus, but one of a marked glycosuria, unaccompanied by polyuria. At the time when these investigations were made, the urine contained about 1·5 % of glucose (estimated by the polarimeter). We have therefore to deal with a urine of normal concentration, in so far as the normal urinary constituents are concerned, which contains glucose in such amounts that more sugar is present than necessary to reduce all the cupric oxide, provided that the usual method of carrying out the test is applied (equal amounts of urine and half diluted Fehling's solution). Now if glucose is added to normal urine in such amounts that the urine contains about 1·5 % of glucose, and Fehling's test is applied to this urine, a deep red, transparent solution is obtained, which represents a colloidal solution of cuprous oxide. The appearance of this red solution instead of a precipitate of cuprous oxide is due partly at any rate to the presence of creatinine, which, as MacLean has shown [1907], is capable of holding cuprous oxide in solution. Other substances, perhaps the colloid material of urine acting as a protective colloid, also contribute to this effect. If this red solution is further heated it darkens to a deep brown solution and deposits a reddish brown precipitate, so that it is possible to imitate to a certain extent the abnormal reduction described at the outset of this paper simply by using a normal urine containing glucose in a certain concentration.

The change of colour from red to black is, however, not so striking in the case of normal urine to which glucose has been added. A much better imitation of the reaction can be obtained with normal urine in the following way.

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Add to half a test tube full of normal urine two drops of chloroform, shake vigorously and apply Fehling's test at once. One obtains then the red, transparent, solution, which darkens without further heating and eventually deposits a black precipitate. If to urine which has been agitated with chloroform, and then allowed to stand so that the chloroform has time to settle, Fehling's test is applied, only the red solution is obtained which does not blacken on further heating. If now to this red solution sugar is added, blackening occurs.

It is therefore clear that this "abnormal" reaction is due to the presence in sufficient quantity of a reducing substance in urine of a normal concentration. The amount of the reducing substance present must be in excess of the amount necessary to reduce all the cupric salt used in the test.

The clinical interest which attaches to this reaction appears to be that it indicates the presence of relatively large amounts of sugar in a urine of normal concentration; it indicates, in other words, a marked glycosuria unaccompanied by polyuria.

Some years ago I obtained from the Infirmary a urine giving exactly the same reaction. Unfortunately, the patient left the hospital before I had time to investigate the condition. But as both this former and the present case were obtained accidentally, when material was being collected for teaching purposes, it seems likely that these "abnormal" reactions may occur much more frequently than would appear from the complete absence in the literature of any similar observation.

Since watery solutions of glucose are capable of reducing cupric salts to metallic copper, it is to be expected that urine from a typical case of diabetes mellitus, where we have a glycosuria accompanied by a polyuria, will also give the reduction to metallic copper. This is, in fact, the case, especially if excessive dilution be avoided by using Fehling's solution undiluted, or if Fehling's solution is diluted with an equal volume of normal urine. If after the appearance of the yellow or red precipitate of cuprous oxide boiling is continued, a marked darkening of the precipitate and of the solution occurs, varying from a light brown to the dark reddish brown copper colour, characteristic of the precipitate obtained by boiling Fehling's solution with a watery solution of glucose. It must be noted, however, that this further reduction occurs only when boiling is continued, and is even then not nearly so striking as the reaction described at the outset of this paper. The variations in colour depend on the amount of glucose in the urine, on the proportion of urine and Fehling's solution, and on the concentration of the mixture both as regards

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the ordinary urinary constituents and the glucose. The darkening of the brick-red cuprous oxide precipitate must have been frequently observed, but it appears to have been ascribed generally to the caramelisation of the sugar by the alkali of Fehling's solution. That this is not the case is evident from the fact that exactly the same reaction is obtained, as stated above, if chloroform instead of glucose is added to the urine.

The reaction obtained with urine containing chloroform is also of interest, because it demonstrates that this "abnormal" Fehling's reaction is due mainly to the cuprous oxide dissolving power of normal urine. For chloroform shaken up with water gives with Fehling's solution only the red precipitate of cuprous oxide, which does not become further reduced to copper even after prolonged boiling.

Summary.

1. A reduction of cupric salts to metallic copper can be brought about by concentrated watery solutions of the reducing sugars. It takes place even more readily when the reducing sugar is present in a urine of normal concentration with regard to the normal urinary constituents, being facilitated by the fact that some of the constituents of normal urine are capable of holding cuprous oxide in solution.

2. Clinically this abnormal reaction with Fehling's solution is of interest, as such a reaction may be taken to indicate a marked glycosuria unaccompanied by polyuria, and not a typical diabetes mellitus.

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IX. ON THE CHOLESTEROL CONTENT OF THE TISSUES OF GROWING RATS WHEN UNDER VARIOUS DIETS.

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It has for some time been known that growing animals are unable to thrive for any length of time on a diet consisting of a mixture of pure protein, fat, and carbohydrate. Stepp [1909, 1911] in a series of experiments on mice attempted to estimate the importance of lipoids in nutrition, and found that food mixtures after extraction with lipoid solvents could not maintain life, but that the extracted matter when added to the food made it once more efficient to do so; he could not obtain this result, however, by adding any known lipoid. A similar series of experiments has been carried out by Osborne and Mendel [1911] on rats. Their earlier work was directed to the question as to whether life could be maintained on a dietary containing a single individual protein, instead of mixtures of proteins such as ordinary normal dietaries comprise, and they were led to answer this question in the affirmative. Maintenance is possible so long as the protein stored is one not deficient in certain amino-acid groupings, but their earlier experiments led these authors to realise the importance of factors other than protein and energy supply and they found that synthetic dietaries which were able to maintain the life of fully grown rats were unable to do so with growing animals, but that a small addition of tissue extracts added to those synthetic diets induced normal growth.

In their later experiments these authors showed that the addition of milk products to the food promoted rapid growth in rats which had remained stationary in weight when on the original artificial diet. The material which was added they called "protein free milk"; it was prepared by removing

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as far as possible by precipitation and coagulation, the proteins from fat free milk, the fluid being then evaporated at low temperatures and the residue ground to powder.

Hopkins [1912] in an extended series of experiments in which groups of young rats were fed upon artificial mixtures of isolated caseinogen, fat, carbohydrates and salts side by side with similar groups which were fed on the same basal dietary with the addition of a minute quantity of fresh milk, has found that in those experiments in which the basal diet contained fully purified material the rats without milk soon failed to grow. When the constituents were less completely purified then slow growth occurred. In all cases the milk addendum, although its total solids only amounted to 4 % or less of the whole food eaten, induced normal and continued growth.

The total energy consumption of these animals was determined and it was shown that the rats upon the purer dietary ceased to grow at a time when their intake was more than sufficient quantitatively to maintain normal growth. The absolute consumption of the animals growing rapidly with the milk addendum was greater than that of those growing slowly upon the basal dietary alone; but the consumption per unit of live weight was in comparable groups nearly the same. It was further shown in these experiments that if growth continued, as upon the less pure basal dietary, the small milk addendum reduced the food consumption necessary for a given weight increment to one half or less.

Cessation in increase of weight upon the pure dietary took place before any failure in appetite, although the consumption might, later, fall to a lower level. It was therefore suggested that any effect of the addendum upon appetite must have been secondary to a more direct effect upon growth processes.

Much discussion has taken place over the question as to what is meant by "growth." A living organism may increase its exterior dimensions without increasing its mass, and, on the other hand, the mass may be increased without any "growth processes" taking place.

When young rats are being fed on a normal diet they certainly grow, by which we mean that the amount of living material in their bodies increases and they increase in size permanently, but still we have to define our terms "grow" and "living material," and we find ourselves arguing in a circle.

The evidence now accumulated, however, points fairly to the conclusion that when "growing rats" are put on to a pure synthetic dietary the phenomena observed during their former growing period cease.

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We may state what has happened either by saying that their "growth processes" have ceased, or by saying that they are being starved and hence cease to grow. It would seem that the latter more accurately states the case.

The synthetic diet seems to lack something which is necessary for the proper utilisation of the other food stuffs by the tissues of the animal.

We may compare the tissues of the rat and the food material to a heap of powder ready to unite on the application of a flame—the hormone present in a normal diet, but absent from a synthetic diet.

The fact then being fairly well established that "growth processes" cease when animals are fed on pure synthetic diets, or in other words, starved, it seemed to be of interest to carry out experiments to endeavour to determine how far variations of the cholesterol content of the bodies of growing rats occur, when fed on various dietaries, especially with regard to the question as to whether the growing organism can manufacture cholesterol when deprived of that substance in the food. It seemed that an answer might be obtained to this question, and to others bearing on the origin, fate and functions of cholesterol in the animal organism, by taking a number of sets of rats, from a common starting point—which was chosen as the period after birth at which the animals commenced to feed themselves—and estimating the total cholesterol content of the bodies of these rats at once, and then comparing the figures obtained with those from other sets of rats, which had been fed for definite periods on various dietaries of known composition. The original set of controls then gives us the normal quantity of cholesterol in the bodies of the rats when they start to feed themselves, whilst the numbers from the other sets give us an indication as to any increase or decrease in the quantity of cholesterol present.

It was determined at the outset to keep all the sets of rats on their particular dietaries until they had either doubled their weight or had been kept for a time equal to that in which a set of rats fed on ordinary bread and oatmeal had doubled their weight, which was found to be just over three weeks.

Owing, however, to the unpalatability of certain of the synthetic and alcohol-and-ether extracted diets, this was found to be difficult as the rats commenced to die after a certain time, so that in some cases I was unable to extend the observations beyond a period of about 14 days.

An endeavour was also made to find a synthetic diet which, by the addition of cholesterol and other substances such as cholesterol esters, lecithin, lemco, etc., would keep the rats in normal growth.

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No synthetic diet was found satisfactory for this purpose and hence no reliable evidence is to hand as to how the cholesterol content of the rats would vary under such conditions.

Method. The animals employed in these experiments were ordinary tame white rats and were mostly bred in the laboratory. They were kept with the mothers until they were able to feed themselves and were then taken in sets of varying number and put into square wire cages of about eight cubic feet capacity. The bottoms of these cages were also of wire, the mesh being of sufficient size to allow the faeces to drop through into a trough placed beneath. Each cage had in it a large pad of cotton wool. It was not thought necessary for the purpose of the experiments to feed the animals separately; they flourish much better when not alone, and furthermore individual variations are eliminated. On the other hand, this plan is open to the disadvantage that if an animal dies the others commence to nibble it.

Diets employed. Altogether 13 sets of rats were analysed; the method of analysis being a modification of Windaus' digitonin process fully described by Fraser and Gardner [1910]. The details of the results are given in Tables I and II.

TABLE I.

Experimental set	Diet	No. of rats	Total weight in grams	Percentage of cholesterol			Weight of cholesterol per rat in grams			Average weight of rats
				Total	Free	Ester	Total	Free	Ester	
1	Starting feeding three weeks old	5	122·45	0·1467	0·0944	0·0523	0·0359	0·0229	0·0130	24·49
2	do. do.	6	133	0·1721	0·1373	0·0348	0·0380	0·0304	0·0076	22·20
3	Bread and oatmeal	4	142·4	0·200	0·144	0·056	0·071	0·051	0·020	35·60
4	Synthetic and milk salts	3	61·0	0·238	0·2122	0·0258	0·048	0·042	0·006	20·30
5	Synthetic and incinerated salts	1	26·8	0·136	0·136	nil	0·0366	0·0366	nil	26·8
6	Synthetic and mixed proteins	3	67·0	0·174	0·174	nil	0·0388	0·0388	nil	22·3
7	Synthetic and milk only	4	117·3	0·2162	0·1198	0·0964	0·0634	0·0351	0·0283	29·3
8	Synthetic and milk and cholesterol	3	76·6	0·5236	0·1608	0·3628	0·1340	0·0429	0·0911	25·50
9	Synthetic and cholesterol	2	34·6	0·428	0·4079	0·0201	0·074	0·0705	0·0045	17·3
10	do. do.	3	52·6	0·5046	0·4821	0·0225	0·0883	0·0812	0·0071	17·5
11	Synthetic and cholesterol esters	2	41·5	0·3606	0·3183	0·0423	0·075	0·066	0·009	20·75
12	do. do.	6	100·0	0·415	0·272	0·143	0·069	0·045	0·024	16·7
13	Ether extracted bread and oatmeal, and cholesterol	4	134·5	0·328	0·3178	0·0102	0·1104	0·1070	0·0034	33·60

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TABLE II.

Expt set	Diet	No. of rats analysed	Average wt. of rat at beginning of experiment	Average weight of rat at end of experiment	Average increase in weight	Length of expts in days
3	Bread and oatmeal	4	18	36·7	18·7	25
4	Synthetic diet and milk salts	3	22·2	20·3	−1·9	16
5	„ „ „ incinerated salts	1	25·7	27	1·3	16
6	„ „ „ mixed proteins	3	20·7	22·8	2·1	15
7	„ „ „ milk only	4	18·2	30·2	12	24
8	„ „ „ milk and cholesterol	3	19·5	26·7	7·2	24
9	„ „ „ cholesterol	2	19·3	18·25	−1·05	13
10	„ „ „ „	3	20·0	18·3	−1·7	15
11	„ „ „ cholesterol esters	2	19·3	20·7	1·4	16
12	„ „ „ „	6	16·6	17·0	0·4	15
13	Ether extracted bread and oatmeal, and cholesterol	4	18·8	34·7	16	31

Set (1). This set consisted of 5 rats which were killed and analysed at the period when they were just commencing to feed themselves. They were then 22 days old, and weighed altogether 126·45 g., the average weight per rat being 25·29 g. The actual minced up material used was 122·45 g. This mass was pounded up with sand and plaster of Paris and allowed to dry. It was then extracted with ether in a Soxhlet apparatus for about 14 days. The ethereal extract was made up to one litre, one half of which was analysed at once, and gave by direct precipitation with digitonin in hot absolute alcohol 0·236 g. of the digitonin compound corresponding to 0·0573 g. of cholesterol. The other half was saponified and gave 0·3698 g. of the compound corresponding to 0·0898 g. of cholesterol. This represents a total cholesterol content of 0·1798 g. corresponding to 0·0359 g. of cholesterol per rat which may be taken as our control standard.

Set (2). This set, consisting of six rats, was treated in a similar way to the last. They were 24 days old and weighed 134·5 g., the average weight per rat being 22·4 g. The actual minced up material used was 133 g.

Total cholesterol content 0·2289 g., or an average of 0·038 g. of cholesterol per rat.

Set (3). *Ordinary diet.* This set consisted of four rats which commenced to feed themselves about 28 days after birth, when their total weight was 72 g. They were then put on a diet of bread and oatmeal and doubled their weight in 25 days. During this period they were given 377 g. of dry bread and 155 g. of oatmeal, the total being 532 g., 447 of which were eaten. The dry bread was soaked in water after being weighed and mixed with the oatmeal.

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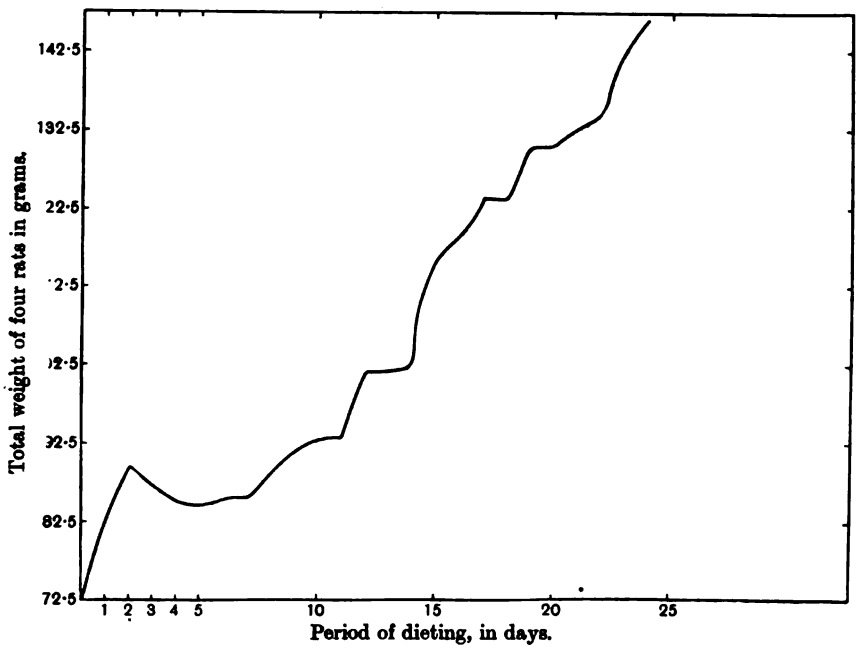
The total weight of the four rats when killed was 146·8 g., their average weight being 36·7 g., whilst the actual weight of material minced up was 142·4 g. Total cholesterol content 0·2846 g.; corresponding to 0·071 g. cholesterol per rat.

This diet of bread and oatmeal was analysed for phytosterol, 0·0235 % of total phytosterol being found. The method employed was to heat the food up with a 10 % solution of alkali on a water bath for some hours, and then after making acid with strong hydrochloric acid, to continue the heating for a similar period. The mass was then treated with ether and estimated



Curve 1. Four rats, ordinary diet (Set 3). Total weight curve.

in the ordinary way. We see now that the increase in the cholesterol per rat over set (1) is 0·071 − 0·0359 = 0·035. Therefore the total increase in cholesterol in the bodies of the four rats is 0·14 g.

Altogether 447 g. of food, containing 0·0233 % of phytosterol were eaten, which means that 0·105 g. phytosterol were ingested. Hence taking into consideration individual variations in the rats and possible errors of experiment there is here no evidence of any synthesis of cholesterol; all the phytosterol contained in the food seems to have been laid hold upon and stored. Curve 1 represents the growth of these rats.

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Set (4). *Basal synthetic diet + milk salts.* This set consisted of six rats which were not started on the dieting until they were 28 days old, when they weighed 133·5 g., their average weight being 22·2 g. These animals were fed on the synthetic diet used by Hopkins [1912] in his extended series of observationson the growth of rats when fed on various diets, which consisted of pure protein, fat, and carbohydrate, with salts added of the same nature and percentage as those found in milk.

Composition of food.

Protein (Hammarsten's pure casein)	22 %
Potato starch	42 %
Cane sugar	21 %
*Lard	12·4 %
Calcium phosphate	1·95 %
Magnesium phosphate	0·35 %
Potassium chloride	0·15 %
Sodium chloride	0·15 %

* The lard was carefully purified and was quite free from cholesterol.

By feeding the rats on this diet I wished to determine firstly whether any growth occurred or not, and secondly whether any increase of cholesterol occurred in the body, none being present in the food. Before administering the food, the protein, starch and sugar were mixed dry, and the lard was then carefully rubbed in by hand and the whole mixed together till of a uniform composition. Thus prepared the mixture represented the dry weight of the food. Each day's ration for the set of rats was weighed out dry, then water was added, a little at a time, the whole being thoroughly mixed so as to make the mass into a light paste, of such consistency that the rats would eat it freely. More food was always given than the rats would eat, and was placed in a shallow vessel so as to be easily accessible; water was placed in a separate vessel. It was found possible to determine the food eaten with great accuracy; the rats spilled out very little, and practically none of the faeces became mixed up with it. The food left over from the previous day was taken out each morning, before feeding, carefully dried and weighed.

During the period of dietary the rats were given altogether 211 g. of food, 175 g. of which were eaten, but in considering these figures one must remember that the rats died progressively, during the experiment, apparently owing to the unpalatability of the food, and by the end of the 16th day only one remained, two having died earlier in the day.

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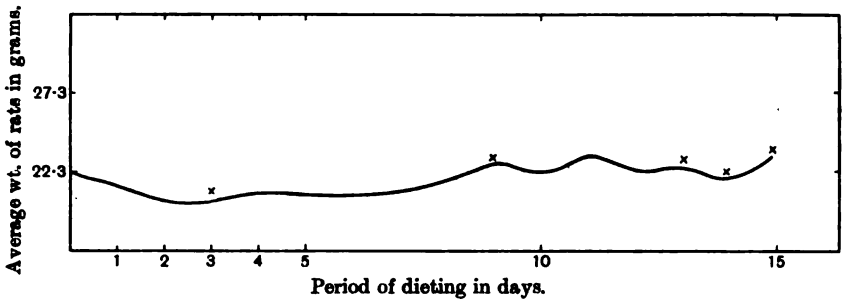
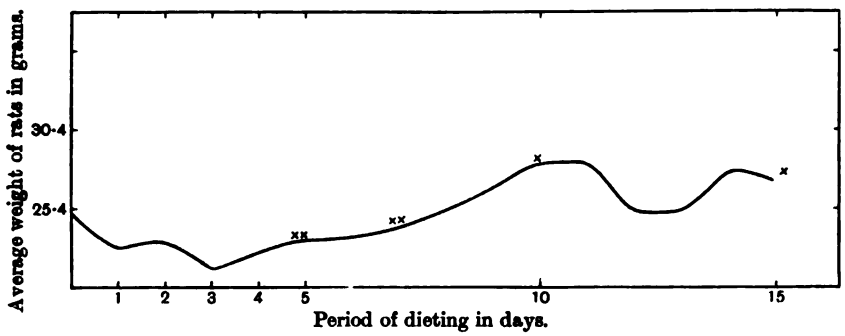
The average weight of these three rats was 20·3 g., and as the average weight of the rats at the commencement of the period of dieting was 22·2 g., we see that no growth has taken place. Curve 2 represents the growth curve of these rats, their average weight being plotted against days of dieting, and the small crosses represent the periods at which successive rats died.



Curve 2. Synthetic diet + milk salts (Set 4). Average weight curve.

Some difficulty was experienced in keeping the rats from nibbling those that died. No nibbling of any importance occurred in this and the next sets, but some did occur in those sets which were fed on an ether and alcohol extracted diet.

Total cholesterol content 0·1454 g., corresponding to 0·048 g. cholesterol per rat. We thus see that this figure is very near that found for set (1) and that no apparent increase of cholesterol has taken place during the period of growth.



Curve 3. Synthetic diet + incinerated salts (Set 5). Average weight curve.

Set (5). This set also consisted of six rats taken from the same litter as the above set, and the rats were put on the special diet 28 days after birth. Their total weight at this time was 154 g., or an average weight of 25·7 g. They were fed on exactly the same diet as set (4) except that the salts used

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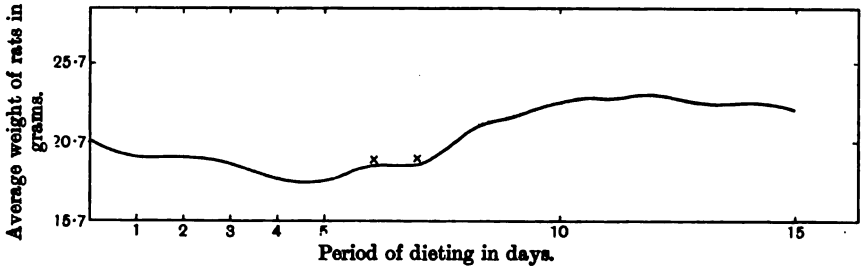
were obtained from incinerating bread and oatmeal. During the period of dieting the rats were given 165·5 g. of food, 125 g. of which were eaten. As in the case of set (4) the rats did not thrive well on this diet, dying at intervals during the course of the experiment, until at the end only one rat was left weighing 27 g., so again we see that practically no increase in weight has taken place.

Curve 3 represents the growth curve of these rats. The last rat was killed on the 16th day and analysed, the actual weight of material used being 26·8 g. Total cholesterol found 0·0366 g. per rat. This figure again very closely approximates to that found for set (1), no increase in the cholesterol content of the organism having taken place.

Set (6). This set consisted of five rats which were fed on a pure synthetic diet as in the case of set (4), but half the protein was replaced by protein obtained from bulls' testicles.



Curve 4. Synthetic (mixed proteins) + lecithin (Set 6). Average weight curve.

This was obtained as follows:

The tissue was carefully ground up and boiled with alcohol, added in the ratio of one litre of alcohol to 300 g. of tissue. The alcohol was filtered off and the boiling repeated. The tissue was then extracted with ether in a Soxhlet apparatus for a fortnight, after which it was again extracted by petroleum ether for a considerable time. In addition 0·5 % of lecithin prepared from bulls' testicles was added. The diet was entirely cholesterol free.

At the beginning of the experiment the five rats weighed 103·5 g., being an average of 20·7 g. One rat died on the 7th day and one on the 8th. On the 16th day, the remaining three rats weighed 68·5 g., being an average of 22·8 g. During this period they were given 150 g. of food, 130 of which were eaten. The actual amount of material analysed was 67 g.

Total cholesterol content per rat 0·0388 g.

Curve 4 shows average growth during the course of the experiment.

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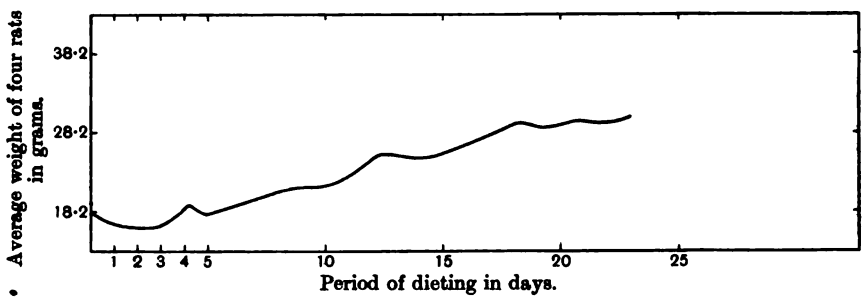
Set (7). This set consisted of four rats born on May 30, 1913, and the animals were put on their special diet on June 30, 1913. This diet was the same as the synthetic diet employed in sets (4) and (5); in addition to which 20 cc. of milk were given each day. We wished to see how the addition of milk alone to the synthetic diet would influence growth and whether the small quantity of cholesterol in the milk would be picked out by the organism and utilised for growth. The weight of the four rats at the commencement of the experiment was 75 g., corresponding to an average of 18·2 g. per rat. At the end of the experiment their total weight was 120·9 g., representing an average of 30·2 g. Each rat had therefore put on during the course of the experiment 12 g., which is a greater increase than in the following case in which cholesterol was also added to the diet, but not so great as in set (13), which had ether extracted bread and oatmeal and cholesterol. During the



Curve 5. Synthetic diet + milk only. No cholesterol (Set 7). Average weight curve.

period of dieting of 24 days, the rats were given 366 g. of food, and 480 cc. of milk, 265 g. of the food being eaten.

Milk contains about 4 % of fat, and milk fat about 0·5 % of cholesterol. The milk given to the rats would thus contain altogether about 0·096 g. of cholesterol.

All the rats remained perfectly healthy during the course of the experiment, none having died. The rats were killed on July 22 and analysed, the actual weight of material used being 117·3 g. Total cholesterol found 0·252 g.; corresponding to 0·063 g. of cholesterol per rat. Now assuming that each rat had in its body the normal quantity (set 1) of cholesterol, viz. 0·0359 g., the total quantity would be 0·144 g. for the four rats. Therefore the total increase for the four rats is 0·252 — 0·144 = 0·108.

The milk given contained as we have seen about 0·096 g. of cholesterol altogether. These figures would therefore indicate that the cholesterol in the milk was greedily picked out and stored in the organism. All the cholesterol

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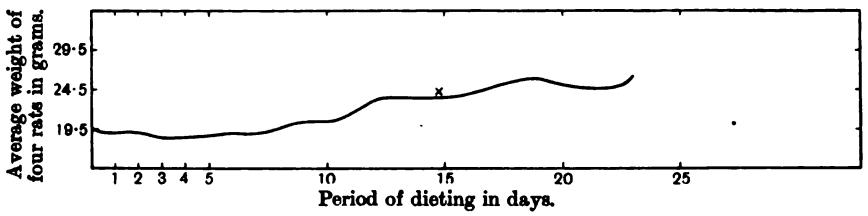
found can thus be accounted for by that contained in the milk, and, taking into account the errors of experiment, there is no evidence of any synthesis having taken place. Curve 5 represents the average growth curve for this set.

Set (8). This set, consisting of four rats from the litter born on May 30, 1913, were put on their special diet on June 30. The diet was exactly the same as the last except that 2 % of cholesterol was added to the food and only 10 cc. milk given each day instead of 20. The dry food after being weighed was carefully mixed with water as in the case of set (4). The object of feeding the rats on this diet was to see how the addition of the milk affected growth, and whether, should normal growth occur, the cholesterol content of the tissues would approach or exceed the normal figure.

The weight of the four rats at the commencement of the period of feeding was 78 g., corresponding to an average of 19·5 g. During the period of dieting the rats were given 338 g. of the food, 230 cc. of milk and 6·8 g.



Curve 6. Synthetic diet (milk salts) + milk + cholesterol 2 % (Set 8). Average weight curve.

cholesterol. As in the previous cases the food left over was carefully dried and weighed and it was found that the rats had eaten approximately 200·7 g. food and 4 g. of cholesterol. The total cholesterol in the 230 cc. of milk added was about 0·048 g. since milk contains 4 % of fat, and the fat 0·5 % of cholesterol.

One rat which met with an accident on the 15th day was killed at once. At the end of the period of dieting the remaining three rats weighed 80·3 g., corresponding to an average weight of 26·7 g. The increase in weight per rat was therefore 7·2 g. during the period of 23 days, which is less than half the average increase of the previous set.

The rats were killed on July 22. Total cholesterol found 0·4012 g., corresponding to 0·134 g. per rat, a figure approximating to that found for set (13), viz. 0·1104 g. per rat. However, growth was much slower on this synthetic diet with milk and cholesterol than on the partly extracted food with cholesterol alone added (set 13). As the total cholesterol found was

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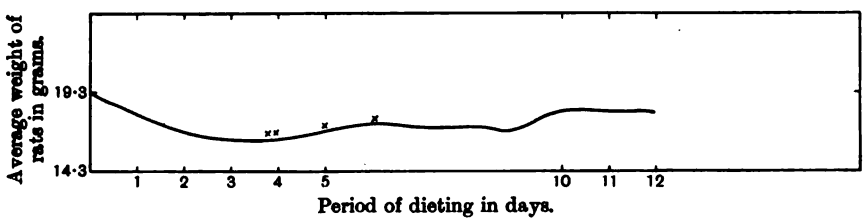
only 0·4012 g. for the three rats and 4 g. had been eaten in the food, it is clear that only about one-tenth of that ingested is stored up.

Curve 6 represents the average weights of these rats plotted against days.

Set (9). This set consisted of six rats which were fed with the synthetic diet used in set (4) to which 2 % of cholesterol was added. It has been seen that in the case of sets (4) and (5) no increase either of growth or of cholesterol content took place on a pure synthetic diet alone, and one wished to see what effect the addition of cholesterol would have on each of these factors. The weight of the six rats at the commencement of the period of dieting was 115·5 g., being an average of 19·3 g. Two rats died on the 4th day, one on the 5th and one on the 6th, but no nibbling whatever occurred as the bodies were removed in time to prevent it. The remaining two rats were killed on the 13th day and analysed. Their weight was then 36 g., being an average of 18 g. No increase in weight had therefore taken place. During the period of dieting the rats were given 116 g. of food, 76 of which were eaten including 1·52 g. of cholesterol. The two rats were killed on the 13th day and analysed. Total cholesterol content for the two rats 0·148 g.; or 0·074 g. per rat.

Now up to the end of the 6th day 35 g. of food had been eaten containing 0·71 g. of cholesterol. Thus the remaining 1·52 — 0·71 or 0·81 g. of cholesterol was eaten by the two rats which were analysed. The amount of cholesterol to be accounted for in their bodies is obviously 2(0·074 — 0·0359) = 0·0762 g. taking 0·0359 as standard, and this is quite covered by the quantity of cholesterol ingested. It will be noted that only about one-tenth of the cholesterol ingested is stored, the remainder being excreted in the faeces.

Curve 7 shows the average weights of the rats during the experiment.



Curve 7. Synthetic diet + cholesterol (Set 9). Average weight curve.

Set (10). This set consisted of four rats whose diet was the same as in the last set but with the following modifications:

(1) The fat employed was manufactured by dissolving glycerol in dry pyridine and adding palmitic chloride. This was allowed to stand over

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night and then poured into water and filtered through a filter pump. Any excess of acid chloride was dissolved out by a dilute solution of sodium carbonate and the residual fat carefully dried.

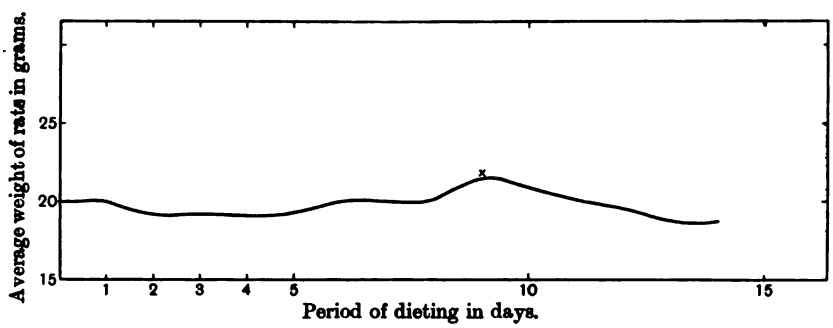
(2) 0·5 % of pure lecithin prepared from bulls' testicles was added to the food, being carefully rubbed in so as to be uniformly distributed.

(3) Half of the pure protein ordinarily employed was replaced by protein obtained from bulls' testicles which was quite cholesterol free (*vide* set 6).

The weight of the four rats at the beginning of the experiment was 81 g., being an average of 20·25 g., and at the end three rats remained whose total weight was 55 g., being an average of 18·3 g., one rat dying on the last day. It was considered that the unpalatability of the synthetic food might be mitigated by flavouring it with meat extract; this was done all through this particular experiment which lasted 15 days. The average weight nevertheless went down, neither the lemco added nor the lecithin seeming to have any pronounced effect on growth or even sufficing to sustain the original weight. During the period of dieting of 15 days the rats were given 144 g. of food and 2·88 g. of cholesterol; 101·3 g. of food were eaten; the cholesterol intake thus being 2 g.

The actual weight of material analysed was 52·6 g. Total cholesterol content 0·2654 g.; or 0·0883 g. per rat. The quantity of cholesterol which the three rats have put on is $3(0{\cdot}0883 - 0{\cdot}0359) = 0{\cdot}572$ g. These rats ingested in their food about 2 g. of cholesterol. All found is therefore accounted for by that contained in the food.

Curve 8 represents the average growth during the course of the experiment.



Curve 8. Synthetic + cholesterol (artificial fat + lecithin and lemco) (Set 10). Average weight curve.

The results so far obtained do not indicate that cholesterol itself has any appreciable influence on the growth of an animal; all that is shown is that when an animal is fed on an artificial diet which does not promote growth

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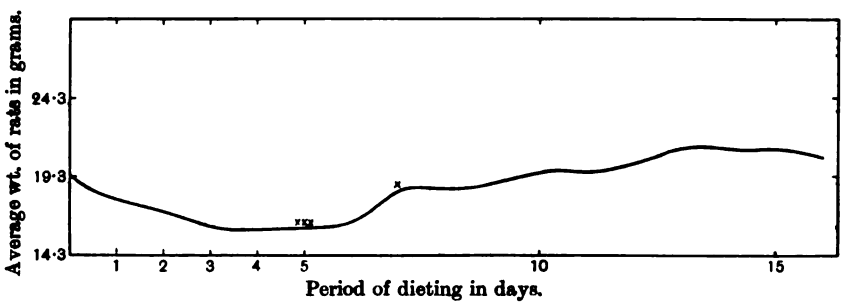
and to which cholesterol is added, no increase of growth occurs, but that part of the added cholesterol is stored bringing the total cholesterol content of an individual rat up to or above that found in the body of a rat fed on an ordinary diet [*vide* set (3)].

Whether the esters of cholesterol have any influence in this direction is a question which has been investigated by adding them to the synthetic diet instead of cholesterol.

Set (11). This set consisted of six rats fed on an ordinary synthetic diet with the addition of 2 % of cholesterol esters. The ester employed was palmitic ester which contains 0·6 of its weight of cholesterol; this is equivalent then to adding 1·2 % of cholesterol.

The weight of the six rats, at the beginning of the experiment, was 116 g., being an average of 19·3 g. per rat. On the 6th day three rats died and one on the 7th day; the two remaining rats remained alive for 16 days in good health when they weighed 43·5 g., being an average of 21·75 g. No lemco was added to the food, and no growth took place. After the four rats had died the remaining two were given 70 g. of food of which 50 were eaten, and the amount which they ate during the previous six days was calculated at 16 g. This represents 66 g. of food containing 1·2 % of cholesterol or 0·792 g. of cholesterol. These two rats were killed on the 16th day when they weighed 43·5 g., or an average of 21·75 g. Total cholesterol content 0·1496 g., or 0·075 g. per rat, representing an increase for the two rats of $2(0{\cdot}075 - 0{\cdot}0359) = 0{\cdot}08$ g.

We have seen that 0·792 g. of cholesterol was ingested so that about one-tenth of this quantity has been stored in the bodies of these two rats.



Curve 9. Synthetic + cholesterol esters (Set 11). Average weight curve.

Curve 9 gives the average weight of these rats during the course of the experiment.

Set (12). This set, consisting of six rats, was fed on exactly the same diet

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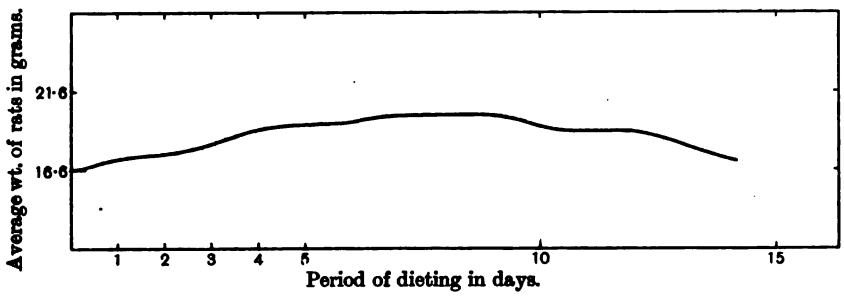
as the last with the addition of 0·5 % lecithin; the fat used was synthesised as in set (10) and half the protein was replaced by the pure protein obtained from bulls' testicles [see set (6)]. On June 24, when the dieting was commenced the six rats weighed 99·5 g., being an average of 16·6 g. For the first five days lemco was used to flavour the diet, at the end of which time the total weight had gone up to 116 g. On the 6th, 7th, 8th and 9th days the lemco was omitted, by which time the total weight was 120·5 g. Lemco was now again added to the diet, but the total weight steadily declined till the 15th day, when it was 105 g. and two rats died. All through the experiment the rats had been quite well, but now began to show signs of diminished vitality; it was therefore decided to analyse them at this point when their average weight, 17 g., was practically what it was at the beginning of the experiment, viz. 16·6 g. The total amount of material analysed was 100 g. Total cholesterol content, 0·416 g., or 0·069 g. per rat. During the 15 days the six rats were given 180 g. of food of which 157 were eaten. This means that about 1·8 g. of cholesterol were also ingested. The total cholesterol put on by the six rats is 6(0·069 − 0·0359) = 0·1986 g., that is to say about one-tenth of that ingested, as was the case in the last set.

Curve 10 gives the average weight during the course of the experiment.



Curve 10. Synthetic + cholesterol esters (artificial fat, mixed proteins, 5 % lecithin, and lemco (Set 12). Average weight curve.

We notice that this set of six rats were kept alive and quite well for a period of 15 days when two died. The rats increased in weight about the middle of the experiment when it was thought interesting to omit the lemco from the diet; after four days from the omission they began to decline in weight, and the re-addition of lemco to the food failed to check the decline which progressed steadily to the end.

It has been an endeavour in these experiments to keep the rats in health for a long period on synthetic diet either with or without cholesterol and to

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note the cholesterol content at the end, but so far complete success has not been obtained.

The results obtained from those sets of rats which were fed on a synthetic diet to which cholesterol and cholesterol esters were added do not go to show that these substances have any effect on the growth of the rats. The inability of a synthetic diet to enable growth processes to go on is evidently not due to the absence of cholesterol.

It seemed interesting, however, to see what would be the result of feeding rats on an ordinary diet of bread and oatmeal which had been extracted with ether, or to which cholesterol was added. Should we find the same results as were obtained in the last four sets or not? Unfortunately, the food material used was not thoroughly extracted as was proved by subsequent examination. 50 g. of the food were heated on a water bath for several hours with a 10 % alkali solution, after which the mixture was made acid and again heated for several hours. The mass was then analysed for phytosterol by the modification of Windaus' method. In the 50 g. taken was found 0·0076 g. of phytosterol, that is 0·0152 %.

From 50 g. of similar food, viz. bread and oatmeal, which had been extracted by alcohol, 0·0074 g. of phytosterol was obtained, i.e. 0·0148 %.

Set (13). This set was fed on bread and oatmeal which had been extracted with ether together with the addition of 2 % cholesterol in the daily ration. We have seen in set (5) how the rats behaved on a fat free diet, and I wished to see what effect the addition of cholesterol would have on the growth and condition of the rats. A litter of 12 rats was born on May 30, 1913. Four of these were put on the special diet on June 30 and during the period of dieting of 31 days, were given 278 g. of bread and 276 g. of oatmeal and 11 g. of cholesterol; 428 g. of bread and oatmeal were eaten and 8·5 g. of cholesterol by the four rats, none of which died. The bread and oatmeal were weighed dry, and, with the cholesterol added, were made into a paste with water of such consistency that the rats would eat it freely. The weight of the four rats at the beginning of the experiment was 75·2 g., representing an average of 18·8 g. per rat. The rats readily ate the mixture and were quite healthy throughout the period of 31 days. At the end of this period their total weight was 138·8 g., corresponding to an average of 34·7 g. per rat. Each rat had therefore gained in weight practically 16 g. Curve 11 represents the average growth during the experiment. There were no deaths and all the rats remained quite healthy. The rats were killed on July 30; the weight of the actual tissue analysed being 134·5 g. Total cholesterol 0·4544 g.,

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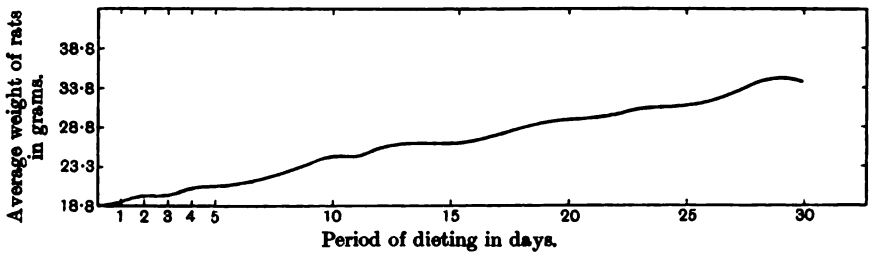
corresponding to 0·1104 g. of cholesterol per rat. It will thus be seen that each rat has put on roughly about 0·0748 g. of cholesterol, again taking 0·0359 from set (1) as our standard. As each rat ate during the course of the experiment about 2 g. of the pure cholesterol given, it will be seen that only a small proportion of that ingested is stored. It is as well to note that the 428 g. of food eaten would contain in all 0·064 g. of sterol which had not been extracted, but this will in no way alter our results.



Curve 11. Ether extracted bread and oatmeal + 2 % cholesterol (Set 13). Average weight curve.

It would seem, in view of the results obtained from sets (9), (10), (11) and (12) which were fed on synthetic diets to which cholesterol and cholesterol esters were given and in which no growth occurred, that the growth in this case is due rather to the fact that the food was imperfectly extracted than to the fact that cholesterol was added.

The results of the experiments detailed above are gathered together in Tables I and II (pp. 81, 82).

Summary of Results.

1. The rats which were just beginning to feed themselves contained in their bodies a normal standard quantity of cholesterol, viz. 0·0359 and 0·038 g. for sets (1) and (2) respectively. The total percentage is 0·1467.

2. After being fed on an ordinary diet of bread and oatmeal for three weeks, the total quantity of cholesterol per rat goes up to 0·071 g., the percentage being 0·2.

3. When the rats are fed on a pure synthetic diet containing no cholesterol, no growth occurs, nor is there any increase in the cholesterol content of the organism over a period of 16 days, and correspondingly there is no loss (sets 4 and 5).

4. The replacement of half of the pure caseinogen used in the synthetic diet by cholesterol-free protein obtained from bulls' testicles, together with

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the addition of lecithin, also causes no increase either in the growth or the cholesterol content of the rats (set 6).

5. When rats were fed on the ordinary synthetic diet, with the addition of considerable quantities of milk, viz. 20 cc. *per diem*, they increased their weight by 66 % in 23 days. The cholesterol in the milk was eagerly picked out by the organism and stored, and the quantity found could be accounted for by that contained in the milk. There was no evidence of any synthesis (set 7).

6. When half the above quantity of milk was given and in addition 2 % of cholesterol, growth was considerably less during the same period of 23 days. About one-tenth of the cholesterol ingested was stored, bringing the total quantity per rat up to 0·1340 g. and the total percentage to 0·5236 (set 8).

7. The addition of cholesterol and cholesterol esters to a synthetic diet, together with lecithin and lemco, did not in any way enable normal growth to proceed. At the end of 15 or 16 days the average weight of the rats fed on a synthetic diet with cholesterol added had slightly decreased, and in the sets which had cholesterol esters added the increase per rat was less than 1 g. during 16 days. In all of these cases the rats stored in their bodies about one-tenth of the total cholesterol eaten with the food (sets 9, 10, 11 and 12).

8. When a diet of bread and oatmeal which had been imperfectly extracted with ether was given together with 2 % of cholesterol, slight growth occurred and the total quantity of cholesterol in the rat was found to be about three times that of set (1). (Set 13.)

General Conclusions.

A pure synthetic dietary is insufficient to enable "growth processes" to go on in young rats, in other words the rats are starved on such a diet and neither cholesterol, cholesterol esters nor lecithin appears to supply the deficiency. Lemco, however, appears to have a beneficial effect in some cases, rats fed on a diet with this addition showing no eagerness to nibble their dead comrades.

There is no evidence that the living organism can manufacture cholesterol, but on the other hand it is a substance which is strictly conserved and readily picked out from a diet in which it is present in only small quantities. When, however, it is present in large quantities in the food then the percentage in the body goes up considerably but only a small quantity of that ingested is actually stored up.

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I take this opportunity of thanking the Grants Committee of the Dixon Fund for help in carrying out this work, and also Mr J. A. Gardner for advice during the progress of the work.

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X. THE SPECIFICITY OF CASEINOGENS. A COMPARATIVE STUDY OF THE CASEINOGENS OF THE COW AND THE SHEEP.

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and HERBERT ERNEST WOODMAN.

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(*Received February 7th, 1915.*)

For many years the question as to whether the caseinogens derived from different species of mammals are one and the same chemical substance has remained undecided.

The general physical and chemical characteristics of the caseinogens precipitated from various milks by dilute acid are so similar that no satisfactory conclusions can be drawn regarding the question of their chemical identity, although some earlier investigators have either supported the hypothesis of chemical identity on the strength of the general similarity of the substances or opposed it on account of observations of slight differences.

Abderhalden and Schittenhelm [1906] compared the amounts of amino-acids contained in the caseinogens of cow's and goat's milk with the result that a striking similarity was demonstrated and the assertion of differences in the quantitative amino-acid composition of the two proteins was not possible.

Elementary analyses of caseinogens coincide so closely that they have been adduced as evidence in favour of the view that the caseinogens of various mammals are identical. The comparison of cow's and sheep's caseinogens, with which this paper is especially concerned, serves to show how closely the analyses agree.

Cow	C 52·96 %	H 7·05 %	N 15·65 %	S 0·758 %	P 0·847 % [Hammarsten, 1883, 1885]
Sheep ..	C 52·92	H 7·05	N 15·60	S 0·771	P 0·809 [Tangl, 1908]

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Nevertheless it has long been suspected that as a matter of fact there may be differences in the structure of various caseinogens, not necessarily in respect to the relative amounts of amino-acids present, but possibly in the actual arrangement and order of these acids within the molecules. This is a reasonable assumption on biological grounds, and biological experiments—the formation of specific precipitins on injection into the blood-stream of pure caseinogen preparations—have given support to this view.

In this paper, however, we bring forward for the first time definite chemical evidence in support of the general assumption of specificity in caseinogens, and proof in particular of a difference in intramolecular structure between cow's and sheep's caseinogens. We have been enabled to do this by a comparative study of the amino-acids derived from the "racemised" caseinogens. If caseinogen be dissolved in dilute alkali and the solution allowed to stand at 37° for about three weeks the value of the optical rotation falls to a practically constant value. The protein is then said to be "racemised" and the amino-acids obtained on subsequent hydrolysis are found to be in some cases optically inactive, in others partially active and in others fully active. Dakin [1912] has shown that in all probability the optical properties of the amino-acids depend upon their positions in the peptide chains. According to his view amino-acids whose carboxyl groups are free and which therefore may occupy terminal positions at the ends of peptide chains, are incapable of racemisation, whilst those whose carboxyl groups are united in actual peptide linkages, and are therefore probably situated within the chains, may undergo racemisation. The optical activity or inactivity of the amino-acids among the hydrolytic products of a "racemised" protein will therefore give some clue as to the original positions of these acids in the protein molecule, although the full interpretation of the significance of these findings is not yet possible.

Accordingly we "racemised" a quantity of sheep's caseinogen with dilute alkali under precisely the same conditions as those employed by Dakin and Dudley [1913] in an investigation of the racemisation of cow's caseinogen. By a comparison of the optical properties of the corresponding amino-acids derived from "racemised" cow's and sheep's caseinogens we hoped possibly to be able to detect structural differences relating to the positions of the respective amino-acids in the molecules of the two proteins. In this we were successful; the following table compares the optical properties of the amino-acids from racemised cow's and sheep's caseinogens respectively:

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	Racemised cow's caseinogen	Racemised sheep's caseinogen
Alanine	inactive (and d ?)	inactive (and d ?)
Valine	*d.* and inactive	*d.* and inactive
Leucine	*l.* and inactive	*l.* and inactive
Tyrosine	inactive	*l.*
Phenylalanine	inactive	inactive
Proline	*l.*	*l.*
Aspartic acid	inactive	inactive
Glutaminic acid	inactive	inactive and *d.*
Arginine	inactive	inactive
Histidine	inactive	inactive
Lysine	inactive	*d.*

It will be seen that, as is to be expected, most of the amino-acids from the two caseinogens display identical optical properties, indicating a general similarity in the molecular structures of the two proteins. But a sharp difference is found in the case of tyrosine. The tyrosine derived from "racemised" cow's caseinogen is completely inactive, that from "racemised" sheep's caseinogen is fully active.

Again, the lysine derived from cow's caseinogen is totally inactive, but the specimen isolated from sheep's caseinogen displayed two-thirds of the full activity. The lysine fraction was originally contaminated with some *l.* proline, which was only removed with difficulty by fractional precipitation of the phosphotungstates. Bearing in mind the possibility that traces of *l.* proline might still cling to the lysine, together with the fact that lysine is not a very stable substance, it seems probable that no racemisation of lysine took place before hydrolysis. Further, we find that a small quantity (about 15 %) of the total glutaminic acid obtained from "racemised" sheep's caseinogen is optically active, while that derived from cow's caseinogen is completely inactive.

Although the significance of these differences cannot yet be fully elucidated, they may be taken at least as direct proof that cow's and sheep's caseinogens are two distinct proteins. Their general structure is undoubtedly very similar and might even be, with regard to amino-acid content, quantitatively practically identical, but small definite differences in the arrangement of the amino-acids within the molecules of the two substances must occur.

We incline to the view that each species of mammal may synthesise its own specific caseinogen, and therefore that every caseinogen is a distinct member of a class of very similar proteins.

One point of difference in the comparisons of the amino-acids from the two caseinogens lies in the fact that in the investigation of cow's caseinogen the "racemised" protein was precipitated from the alkaline solution after

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"racemisation" and hydrolysed separately (except in the isolation of tyrosine), whereas the alkaline solution of sheep's caseinogen was hydrolysed as it stood, thus including the products of the hydrolysis which proceeds simultaneously with "racemisation." We do not, however, consider that this procedure vitiates our present results because it has been shown [Dakin and Dudley, 1913] that the amino-acids from "racemised" caseose, the main hydrolytic product during the process of "racemisation," are identical with those of "racemised" caseinogen, and that the quantity of free amino-acids formed under the experimental conditions of "racemisation" is very small.

Fortunately our most definite finding, the difference between the two specimens of tyrosine, is in any case not affected by this possible criticism, since in the case of both caseinogens the entire alkaline solutions were hydrolysed as they stood after digestion, and worked up in exactly the same way.

We propose to extend these investigations to the study of the relationships between other proteins.

In conclusion we desire to thank Dr H. D. Dakin for permission to apply the method of protein racemisation to problems of this nature.

Experimental.

Preparation of sheep's caseinogen.

The caseinogen was precipitated from the "separated" milk by means of dilute acetic acid. The best conditions for precipitation were found to be as follows.

A 0·06 % (vol.) solution of acetic acid was prepared in a large glass vessel and for each litre of this solution 50 cc. of milk were added with vigorous stirring. The caseinogen, which flocked out almost immediately, was allowed to settle and the clear liquid was siphoned off. The caseinogen precipitate was washed with distilled water until free from soluble proteins. If tap water is used the preparation becomes discoloured. After filtering on a large Buchner funnel the caseinogen was ground up in a mortar with an alcohol-ether (4 : 6) mixture, filtered, ground up again with ether, filtered and dried in vacuo over sulphuric acid.

Racemisation of sheep's caseinogen.

This was carried out exactly as in the case of cow's caseinogen [Dakin and Dudley, 1913], 10 % solutions of the caseinogen in $n/2$ sodium hydroxide being digested at 37° for 20–24 days. The fall in rotation and the amount of rotation were of a similar order to those recorded for cow's caseinogen.

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Isolation of Amino-acids.

Tyrosine. 50 g. sheep's caseinogen were racemised. The alkaline solution was then hydrolysed with hydrochloric acid, the solution filtered and evaporated in vacuo at 45°. The residue was then taken up in water, boiled with animal charcoal and filtered. The solution, rendered slightly alkaline with ammonia, was concentrated and tyrosine was allowed to crystallise. The crude tyrosine was filtered off, washed with small quantities of hot glacial acetic acid, and then dissolved in hydrochloric acid. The solution, after treatment with animal charcoal, was neutralised with ammonia and on cooling tyrosine crystallised in characteristic form. It was found to be fully *laevo*-rotatory. It was compared with a specimen of pure *l.* tyrosine prepared by the pancreatic digestion of cow's caseinogen. In 4 % HCl solution the two specimens showed identical specific rotations: − 11·60° and − 11·65°.

400 g. sheep's caseinogen were racemised in the usual way and then hydrolysed with hydrochloric acid for investigation of the amino-acids by the esterification method. Levene's modification of Fischer's method [Levene and van Slyke, 1909] was employed.

Alanine. A specimen of pure *i*-alanine was obtained by fractional crystallisation. Other fractions containing alanine and valine were recovered which possessed a *dextro*-rotation. They may have been mixtures of *d.* alanine and *d.* valine, or possibly of *i.* alanine and *d.* valine.

Valine was isolated in mixtures of *i.* and *d.* valine containing over 50 % of the active form. The *dextro*-variety was also found in leucine-valine mixtures and in valine-alanine fractions.

Leucine was obtained in the pure state almost inactive, and fractions of *l.* leucine containing only very small amounts of *d.* valine were also separated.

Phenylalanine was readily obtained in the pure state and was found to be totally inactive.

Proline was isolated and displayed the partial racemisation which always occurs as a result of the process of separation. Its activity was such as to render it probable that no racemisation had occurred before hydrolysis.

Aspartic acid was isolated in the inactive form. None of the active variety could be detected.

Glutaminic acid was obtained in large quantity and proved to be a mixture of the inactive acid with about 15 % of the *dextro*-form.

Arginine, histidine and lysine were isolated by the method of Kossel and

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Kutscher. 150 g. sheep's caseinogen were racemised for this purpose. Arginine and histidine were both inactive. The lysine fraction displayed a definite *laevo*-rotation when first isolated. By fractional precipitation of the phosphotungstates a specimen of *l*. proline was removed and found to be responsible for the original *l*. rotation. The lysine, examined as the dihydrochloride, now displayed a specific rotation of + 9·4°. It was identified by the preparation of the characteristic picrate.

Summary.

By comparing the optical properties of the amino-acids derived from "racemised" cow's and sheep's caseinogens it is shown that the intramolecular arrangement of the amino-acids in these two proteins is not identical, and it is considered probable that each milk-producing species may synthesise its own specific caseinogen.

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XI. LIGNOCERIC ACID FROM "CARNAUBON."

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Nearly ten years ago Dunham [1905] discovered that ox kidneys contained a substance resembling the so-called "protagon" which Liebreich had found in brain. On comparing the two substances prepared by identical methods from brain and from kidneys (extraction of the fresh tissue with 85 % alcohol at 45°, or with boiling alcohol after the coagulation of the proteins by 5 % sodium sulphate solution), he found that "the substance from the kidney contains distinctly more nitrogen and phosphorus than that from the brain," but that "the cleavage products show that all of these substances belong to the same group."

Later on, however, Dunham [1908] succeeded in isolating from the kidney-"protagon"[1] an acid of the composition $C_{24}H_{48}O_2$ which he considered identical with carnaubic acid. As this fatty acid of vegetable origin had not been previously found in animal tissues, Dunham and Jacobson [1910] gave the name "carnaubon" to this "protagon"-like lipoid of the kidney. From their hydrolysis experiments they came to the conclusion that "carnaubon" represented a triamino-monophosphatide in which an amino-galactose is connected with three fatty acids (carnaubic, stearic and palmitic acid) and with phosphoric acid, whilst the latter is still further connected with two choline groups.

Maclean [1912], in his investigation of the phosphatides of the kidney, obtained a similar lipoid from horse kidney, but he found that the substance contained a water-soluble nitrogenous impurity and that after its removal

[1] Under this name the substance has already passed into the literature. [See Oppenheimer, 1909.]

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the nitrogen : phosphorus ratio had fallen from 3 : 1 to 2 : 1. The substance resembled the diamino-phosphatide sphingomyelin in its general properties, but it still furnished a reducing sugar on hydrolysis.

In the course of some unpublished experiments on the products of hydrolysis of brain-"protagon," Rosenheim noticed that the mixture of fatty acids contained one which closely resembled the carnaubic acid described by Dunham as a typical constituent of "carnaubon." Rosenheim found further [1913, 1, 2] that of the constituents of "protagon" the galactoside kerasin yielded this acid (which he identified with lignoceric acid) as its characteristic fatty acid, whilst the phosphatide sphingomyelin also contained it in smaller amounts. Similar results were obtained simultaneously by Levene [1913] and by Thierfelder [1913], who, however, called the acid "kerasinic acid."

In the light of this experience it seemed of interest to reinvestigate the fatty acids of "carnaubon." We prepared therefore this lipoid from ox kidneys as well as from horse kidneys according to Dunham and Jacobson's directions and hydrolysed the products by alcoholic sulphuric acid. We obtained in confirmation of Dunham's results a fatty acid of the composition $C_{24}H_{48}O_2$, but we were able definitely to identify this acid with lignoceric acid.

There seems therefore no further justification for the retention of the name "carnaubon" for the "protagon"-like lipoid of the kidney[1].

We were unable to find palmitic and stearic acid amongst the products of hydrolysis of "carnaubon," but isolated instead a fatty acid of a higher melting point (94°); this was characterised by its acetyl-derivative as the hydroxy-acid, phrenosinic acid ($C_{25}H_{50}O_3$), which is the fatty acid of the galactoside phrenosin[2].

Moreover, we found that the basic products of hydrolysis contained not only choline, which Dunham and Jacobson considered as the only base present, but also an ether-soluble base identical with sphingosine, a base also obtained on hydrolysis of brain-"protagon" [Rosenheim and Tebb, 1907].

These facts cannot be made to harmonise with the views of Dunham and Jacobson as to the constitution of "carnaubon." If we consider in conjunction with the above results the fact that the elementary composition of

[1] It may be pointed out in this connection that the uniformity of "carnaubic" acid itself has recently been made doubtful. [Röhmann, 1905, Matthes and Sanders, 1908, Lewkowitsch, 1913.]

[2] Palmitic and stearic acid were not satisfactorily identified by Dunham and Jacobson (no melting points are given) and their combustion results are in fair agreement with those required by phrenosinic acid, containing an admixture of lignoceric acid.

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different preparations of "carnaubon" is extremely variable[1], we come to the conclusion that the kidney-"protagon" represents a variable mixture of lipoids, mainly of galactosides (cerebrosides) and sphingomyelin. In distinction from brain-"protagon" it seems that sphingomyelin preponderates in kidney-"protagon."

Experimental.

"Carnaubon" was prepared from ox kidneys according to Dunham and Jacobson's directions. From 8 kg. fresh ox kidneys were obtained 2·93 g. of twice recrystallised product, which after treatment with water and two further recrystallisations yielded 1·0 g. of carnaubon. In another preparation horse kidneys were employed, about 2 kg. being worked up at a time until 2·5 g. of "pure" carnaubon were available.

Ethyl ester of lignoceric acid.

0·7 g. of carnaubon (ox) was hydrolysed by boiling for six hours under a reflux condenser with 35 cc. absolute alcohol containing 8 % conc. sulphuric acid. The ester crystallised out on cooling in glistening white crystals, which were filtered off on hardened filter paper, washed with cold alcohol and dried in vacuo. The crystals could be easily separated from the paper after drying as a felted silky sheet, which weighed 0·11 g. The ester was twice recrystallised from acetone and finally dried in vacuo at 35°.

It melted at 56° and solidified at 54°. Mixed with lignoceric acid ester from wood paraffin[2] it showed exactly the same melting and solidifying point.

The quantity of the ester available was sufficient for two micro-combustions.

(1) 13·050 mg.; 37·600 mg. CO_2; 15·175 mg. H_2O.
(2) 8·465 mg.; 24·275 mg. CO_2; 9·840 mg. H_2O.

	(1)	(2)	Calc. for $C_{23}H_{47}COOC_2H_5$
C	78·58 %	78·21 %	78·71 %
H	13·01 %	13·01 %	13·22 %

2 g. of carnaubon (horse) were hydrolysed by boiling with 100 cc. absolute alcohol containing 5 % conc. sulphuric acid as before. 0·47 g. of the ester was obtained, which was recrystallised from acetone as above. It showed a

[1] The elementary composition of "carnaubon" obtained by Dunham [1905], Dunham and Jacobson [1910] and Maclean [1912] shows the following variations:

C %	H %	P %	N %	S %
65·61–68·19	11·00–12·37	2·02–3·44	2·55–3·26	0–0·82

[2] Prof. Hell, the discoverer of lignoceric acid [1880], kindly put at Dr Rosenheim's disposal some years ago samples of his original lignoceric acid and its ethyl ester, for which we wish to take this further opportunity of thanking him.

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melting point of 56·1°–56·4° and solidified at 53·6°. A mixture with Hell's ethyl ester of lignoceric acid melted at the same temperature.

Molecular weight estimation. 0·3835 g. was saponified by boiling for three hours with 16·04 cc. 0·1 N alcoholic potash. The excess of potash was then titrated back by means of 0·1 N HCl. 9·71 cc. of 0·1 N KOH had been used up. M.W. = 395. Calc. for $C_{23}H_{47}COOC_2H_5$: 396.

Lignoceric acid.

The potassium salt of the acid was prepared from the ester by saponification with alcoholic potash. The salt was well washed with alcohol and acetone, dried in vacuo and the acid set free by dilute sulphuric acid. The free fatty acid was repeatedly washed with water by fusing its watery suspension on the water-bath. It was then extracted with ether, the ether solution washed with water in a separating funnel, filtered through dry filter paper and evaporated. The white crystalline residue of the free acid was recrystallised from a mixture of acetone and petroleum ether. It was obtained in silky white crystals, which assumed a wax-like consistency when compressed in an agate mortar.

It melted sharply at 81° and solidified at 74·2°–73·5°. Samples were mixed with Hell's lignoceric acid, further with lignoceric acid prepared from arachis oil [Kreiling, 1888] and with lignoceric acid from kerasin [Rosenheim, 1913, 1, 2].

The melting points of the mixtures are given in the following table.

		Melts at	Solidifies at
Acid from "carnaubon" mixed with	Lignoceric acid (Hell)	81·1°	74·3–73·4°
	Lignoceric acid from arachis oil	81·0°	74·5–73·5°
	Lignoceric acid from kerasin	81·0°	74·4–73·5°

Molecular weight estimation. 0·2192 g. of the acid, dissolved in benzene and petrol ether, was titrated with 0·1 N alcoholic potash (phenolphthalein as indicator) and required 6·00 cc. 0·1 N KOH.

M.W. = 365. Calc. for $C_{24}H_{48}O_2$: 368.

From the potassium salt obtained in the above molecular weight estimation the acid was again set free as before and after a further recrystallisation showed the same melting point (81·1°) as before.

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Lead salt.

0·1 g. of the acid, twice purified by means of the potassium salt, was dissolved in methyl alcohol and precipitated with a solution of lead acetate in methyl alcohol. A drop of dilute methyl alcoholic ammonia was added to complete the precipitation. The voluminous white lead salt was filtered, washed with hot acetone and dried in vacuo. It was obtained as a white heavy powder which melted at 117°. Hell and Hermanns [1880] gave the melting point of their lead lignocerate as 117°.

	0·1289 g. gave 0·0412 g. lead sulphate	Calc. for $(C_{24}H_{47}O_2)_2$ Pb
Pb:	21·83 %	21·99 %

For the purpose of isolating the other products of hydrolysis, the original alcoholic solution (after the filtration of the ester of lignoceric acid) was diluted with water and the alcohol evaporated on the water-bath. An oily semi-solid substance gradually collected on the surface, and solidified on cooling. This substance was found to consist of a mixture of phrenosinic acid (and its ester) and sphingosine sulphate, which were separated by means of ether, in which the sulphate of the base is insoluble.

Phrenosinic acid[1]. After evaporation of the alcohol the opaque watery fluid was extracted with ether. The ether residue was dissolved in alcohol and neutralised with alcoholic potash. An insoluble potassium salt settled down, from which the fatty acid was set free by dilute sulphuric acid. The free acid was taken up in ether and afterwards recrystallised twice from acetone. It crystallised from alcohol in the characteristic "mamillary" masses which have already been described by Thudichum and later by Thierfelder. The substance melted at 92° and after two recrystallisations at 93°–94°. Levene and Jacobs [1912] have shown that phrenosinic acid exists in two optically isomeric forms, of which the inactive one melts at 82°–85° (Thudichum's neurostearic acid) and the dextro-rotatory form at 106°–108° (Thierfelder's cerebronic acid), whilst a mixture of the two melts at 92°–95°. The acid obtained by us from "carnaubon" is evidently a mixture of the two isomeric forms. It is interesting to note that Grey [1913] also isolated a phrenosinic (cerebronic) acid of the melting point 91° from the mixed fatty acids of brain.

Acetyl-phrenosinic acid. The free acid was boiled for two hours with an excess of acetic anhydride. After cooling, the crystalline acetyl product was

[1] Phrenosinic acid = Thudichum's "neurostearic" acid = Thierfelder's "cerebronic" acid. [See O. Rosenheim, 1914.]

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filtered, washed with alcohol and dried in vacuo over soda lime. It was recrystallised from petrol ether and melted at 64°–65°. It solidified at 62°–63°. According to Levene and West [1913] the acetyl derivative of the inactive phrenosinic acid "melts at 55·5°–56° and solidifies at 53°–54°. The active form would melt higher."

Sphingosine. After the extraction of the phrenosinic acid by ether, the somewhat gelatinous precipitate of sphingosine sulphate left in the aqueous hydrolysis fluid was filtered and washed. The substance was dissolved in alcohol and the base set free by means of alcoholic potash. After dilution with water, it was extracted with ether and remained after removal of the ether as a partly crystalline mass. The free base was converted into the sulphate by neutralising its alcoholic solution with alcoholic sulphuric acid. The insoluble sulphate was filtered off and recrystallised twice from alcohol. It crystallised in needles arranged in rosettes and was finally obtained as a white crystalline powder. On heating it gradually decomposes and melts at 243°–244°, effervescing at 250°. Thierfelder [1904] states that it decomposes with effervescence between 240°–250°. The substance gives with sulphuric acid and sucrose a typical Pettenkofer's reaction and yields nitrogen with nitrous acid. A quantitative estimation in van Slyke's micro-apparatus was unfortunately lost owing to unexpected excessive frothing.

The final aqueous hydrolysis fluid gave Molisch's test and contained a reducing sugar (galactose?) and phosphoric acid. The presence of choline was demonstrated by the periodide test.

We are at present engaged with the preparation of larger quantities of the material, in order to separate the galactosides from sphingomyelin by the pyridine method [Rosenheim, 1914] and to investigate further their cleavage products.

The expenses of this research have been in part defrayed from a grant to Dr O. Rosenheim from the Government Grant Committee of the Royal Society.

Summary.

1. The fatty acid $C_{24}H_{48}O_2$ obtainable on hydrolysis of "carnaubon" is not carnaubic acid, but is identical with lignoceric acid.

2. Phrenosinic acid ($C_{25}H_{50}O_3$) and the base sphingosine ($C_{17}H_{35}NO_2$) were isolated as hitherto unrecognised cleavage products of "carnaubon."

3. There is no justification for the retention of the name "carnaubon" for the lipoid mixture which can be obtained from kidneys by extraction with alcohol.

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XII. RESEARCHES ON THE INHIBITION PRODUCED BY CERTAIN SERA ON THE COAGULATING POWER OF RENNET.

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Introduction.

Among the numerous papers of the last 20 years dealing with the reaction of rennet and its antibodies, those of Morgenroth [1899], Korschun [1902] and Hedin [1906, 1909] have been of particular interest.

In order to compare the effects of enzymes and toxins Morgenroth immunised goats against rennet and was thus able to make a rennet antiserum, which had a strong inhibiting effect upon the activity of rennet. That this inhibiting effect was due to antibodies, formed in the antiserum during the immunisation, and not to some substance in the normal goat serum, is demonstrated by Morgenroth in the following manner. Fresh milk and milk mixed with normal goat serum require exactly the same amount of rennet for their coagulation, whereas milk mixed with the antiserum in the same proportions as milk with normal goat serum only coagulates in presence of a much greater quantity of enzyme. Before Morgenroth's investigations were carried out, Hammarsten and Rödén [1887] had shown that a similar inhibiting effect may be obtained when milk is mixed with normal horse serum. In addition to this, Briot [1900] investigated the blood serum of other animals and showed that the serum of all the species examined had a more or less distinct effect on the coagulating power of rennet. In addition Briot immunised rabbits with rennet and obtained an antiserum similar to that which Morgenroth had made in goats.

In later investigations on the reaction of rennet and its antibodies the preparation of an antiserum has, however, not been attempted, because normal horse serum has a strong inhibiting effect on rennet, which was supposed to be due to a true antibody, and this obviated the necessity of preparing one. Korschun adopts this view in his publication of 1902. His

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researches, which are of considerable interest, bring him to the conclusion that normal horse serum contains two substances with inhibiting action on rennet, one having a large range of action, from 20°–37°, and the other being especially active at 37°. Korschun calls this second substance "pseudo-antilab" (pseudo-antirennet).

A more detailed account of a few of Korschun's experiments will show what led him to accept the above-mentioned theory. In order to determine the coagulating power of milk, Korschun uses the quantitative method, introduced by Morgenroth, which is undoubtedly the most reliable we have. In contradistinction to the others, this method does not indicate the coagulating power of the milk as a function of the time needed for the coagulation of a certain quantity of milk by a constant quantity of rennet, but by the minimal amount of the enzyme, which is sufficient to coagulate this quantity of milk. This method, however, cannot give reliable results, when the coagulation of the milk, that is, the transformation of the colloid caseinogen into precipitated casein is brought about in the usual way, i.e. by heating the milk-rennet complex to about 35° until the transformation has taken place. For by this heating a rather considerable amount of rennet may be destroyed without it having any effect on the caseinogen. Morgenroth's method therefore makes the transformation of the caseinogen take place at lower temperatures (0°–8°), which do not injure the rennet, so that even very small quantities may remain active for a long time. At the low temperature, however, the casein formed is not precipitated and it is therefore necessary to raise the temperature of the milk-rennet complex to about 35°, when all the caseinogen has been transformed. At 35° the precipitation takes place rapidly, often instantaneously. The transformation of caseinogen into casein proceeds only slowly at 0°–8°, and to ensure that the reaction should come to an end, the milk-rennet complex has to be kept at low temperature for 12–16 hours.

Morgenroth's quantitative method is very exact, and the same milk tested at various times always indicates the same coagulating power, which is by no means the case, when this power is determined by the time which is taken for the coagulation of a certain quantity of milk with a constant quantity of rennet.

Korschun's method of testing the power of his serum is also much more reliable than the methods previously used. In these earlier methods various quantities of serum and milk were mixed and the time noted which a certain quantity of rennet required for coagulating the mixture of milk and serum at the optimal temperature of the reaction (i.e. 35°). The inhibiting power of

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the serum was then expressed by the difference between the time taken for the above-mentioned process and the time required for the coagulation of the same quantity of milk without serum by the same amount of rennet. Such a method, however, only gives quite a rough idea of what really happens, because by mixing serum and milk, both these substances act on the rennet, when this is being added to the mixture, and a part of the enzyme will therefore be adsorbed by the milk, and thus rendered incapable of acting with the serum. Hedin proved, in fact, some years later, that serum has the greatest inhibiting effect on rennet, when the serum and rennet are previously mixed, and milk added to them only when the action of the serum has come to an end. The same way of testing the action of serum on rennet is used in Korschun's experiments. Increasing amounts of rennet solution are added to a constant quantity of serum, and after this mixture has been kept for 15 minutes at 20°, 10 cc. of milk are added. The samples are kept all night (12–16 hours) in the ice room, and then moved to 35° in order to determine in which samples the caseinogen of the milk has been transformed into casein. The tube, which contains the greatest quantity of rennet, and is still liquid, indicates the limit of the inhibiting power of the serum. The degree of this inhibiting power is given by the ratio of rennet used to the smallest amount of enzyme, just sufficient to coagulate 10 cc. of the same milk without serum. For example, if 10 cc. of normal horse serum are able to neutralise the effect of 0·03 cc. of a rennet solution on 10 cc. of milk, whilst 0·0006 cc. rennet is sufficient to coagulate 10 cc. of the same milk without serum, then the inhibiting power of this serum is equal to 50.

Using the above-mentioned method Korschun examines the inhibiting power of fresh normal horse serum, of dialysed and of heated horse serum, and finds that dialysis does not decrease the inhibiting power. By heating the serum, however, its effect is lost. He shows, moreover, that a horse antiserum, made by immunising a goat against horse serum, neutralises the inhibiting effect of the normal horse serum. From these facts he concludes that the inhibiting effect of the normal horse serum is due to a true antibody.

Experimenting with horse serum Korschun observed that this serum neutralises more rennet than might be expected, when the rennet-serum complex is kept at 35° or 40° instead of at 20°, and that the increase of the inhibiting power at 37°–40° is greater, up to a certain point, the longer the rennet-serum mixture is kept at the higher temperature. At 20° on the contrary no such increase in the inhibiting faculty seems to take place. He assumes therefore that in addition to the true antibody, the normal horse

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serum contains a second inhibitory substance, the pseudo-antirennet, which is only active at higher temperatures.

As the dialysed serum does not show the greater effect on rennet at higher temperatures, which is shown by normal horse serum, the pseudo-antirennet must according to Korschun be dialysable. On the other hand, a heated horse serum has a strong inhibiting effect at 37° and no effect at 20°; the pseudo-antirennet appears therefore to be highly thermostable.

Hedin's researches point in quite another direction than Korschun's. In a number of publications he discusses the inhibiting influence of different substances on the activity of trypsin and rennet. In regard to rennet he points out that the intensity of the inhibiting effect depends on the order in which rennet, serum and milk are mixed. As already mentioned, he finds that the greatest effect is obtained when rennet and serum are first mixed, and the milk added to this complex, when the action of the serum has been brought to an end. Furthermore, Hedin shows that the longer the complex of rennet and serum is kept, before the milk is added, the greater up to a certain point is the amount of rennet neutralised, and that the maximum amount of the enzyme, which the serum is capable of destroying under the conditions observed, is different for different temperatures, the serum being more active at a higher than at a lower temperature. The concentration of the serum, however, does not seem to have any influence at all on the amount of rennet destroyed, and the inhibiting effect therefore is equally intense, whether the serum used is diluted or not.

Particular interest attaches to Hedin's observation that charcoal affects the rennet in the same way as normal horse serum, thus tending to indicate that the inhibiting effect of this serum is of a less complicated nature than was supposed by Korschun, and that it may possibly be due to a single character of the serum—its adsorbing quality. The further question then arises as to whether the inhibiting effect of the artificially produced rennet antiserum is of a nature similar to that of the normal horse serum, or due to the presence of a true antibody, formed in the serum during the immunisation.

In the experiments discussed below I have examined the inhibiting effect of normal horse serum, in order if possible to obtain agreement between Korschun's and Hedin's theories, which, as already shown, differ in various respects. At the same time the effect of the antirennet of normal horse serum has been compared with the inhibiting power of the antirennet formed in a rabbit serum after an immunisation, with the view of establishing their identity or otherwise.

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I. Researches on the Inhibiting Substances of Normal Horse Serum.

(a) *The true antibody of horse serum and its mode of action.*

The capacity of neutralising a maximum amount of rennet in a very short time (a few minutes) is, according to Korschun, one of the most distinct qualities of the true antirennet, found in normal horse serum. Korschun therefore determines the inhibiting effect of this antibody by mixing horse serum and rennet solution, keeping the complex at 20° for 15 minutes, and measuring the remaining amount of active rennet.

In contradistinction to the action of the true antibody, the inhibiting effect of the so-called pseudo-antirennet only takes place after some time. For the determination of the effect of this substance therefore it is necessary to keep serum and rennet in contact with each other for at least three hours.

From Korschun's work it is evident that the true antibody is fully active at 20°, whereas the pseudo-antirennet does not show any distinct inhibiting effect at this temperature. Furthermore, as the effect of the true antibody does not increase proportionally to the time during which the serum acts on the rennet, it might be expected that the normal horse serum would behave similarly at 20°.

Experiment 1. (Table I.)

In this, as in all other experiments mentioned in this paper, Morgenroth's quantitative method has been used to determine the amount of rennet not neutralised by the serum in question. 10 cc. of milk were added to the rennet-serum complex, after this had been kept for the time required at 20°. The samples were then placed at a low temperature (0°) over night, and the following morning heated in a water-bath to 35°. The caseinogen of the milk was regarded as unaltered in those samples which remained liquid after half an hour at 35°, i.e. in such samples there had not been rennet enough left to transform the caseinogen into casein.

The minimal quantity of rennet solution required for coagulating 10 cc. of the milk used was 0·0015 cc.

The rennet solution in question was a standard solution, made at The Swiss Institute for Dairy Bacteriology, Liebefeld near Berne, according to Morgenroth's instructions. In cases where the amount of rennet used was less than 0·015 cc. the standard solution was diluted (1–10) with saline solution.

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The milk used for the experiments was a mixed milk saturated with chloroform, and curdling as mentioned above by the addition of 0·0015 cc. of rennet.

The horse serum in question was a normal horse serum, the durability of which was increased by the addition of some thymol crystals.

All experiments were made in test-tubes, the inner wall of which was covered with a thin film of paraffin in order to prevent alkali from the glass affecting the rennet-serum complex. All liquids used for the experiments were also kept in paraffined tubes, or tubes of Jena-glass.

Table I shows the inhibiting power of normal horse serum at 20°.

TABLE I.

	Time during which the rennet-serum complex is kept at 20° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·1 cc. of normal horse serum	0·04 cc.	0·04 cc.	0·06 cc.	0·07 cc.	0·08 cc.	0·09 cc.	0·09 cc.	0·09 cc.	0·09 cc.

The comparatively large amount of rennet neutralised at the moment of admixture, and which is not increased within the following 15 minutes, represents an actual change within this short period, and is not due to a continuation of the process when the samples were stored at 0° over night. The quantity of rennet neutralised at 0° is actually very small. An experiment, in which liquids, glasses, pipettes, etc., were kept at 0°, showed that the greatest quantity of rennet, neutralised by 0·1 cc. of normal horse serum under these circumstances, amounted to 0·005 cc.

Table I shows that the time during which the serum acts on rennet is not without importance for the inhibiting power of normal horse serum at 20°. This agrees fully with Hedin's observations. According to Korschun's views, however, the objection may be raised, that the effect of the pseudo-anti-rennet sets in at 20°. To examine the inhibiting power of the true antibody in the horse serum, it is therefore necessary to use a method other than that employed in Experiment 1.

Korschun has shown that the two inhibiting substances in the normal horse serum can be separated by dialysing the serum. As distinct from the true antibody, the pseudo-antirennet is dialysable. A dialysed horse serum should therefore give a measure only of the genuine antibody's mode of action.

As seen in Table I normal horse serum instantaneously neutralises a considerable quantity of rennet—0·04 cc. This quantity undoubtedly is

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neutralised by the true antibody. A dialysed serum should therefore be capable of destroying a corresponding quantity of rennet in an equally short time, and thereby have reached the maximum of its inhibiting power. As can be seen from Tables II and III, this is by no means the case.

In these tables the results are given of two experiments, made with two samples of horse serum, the one dialysed longer than the other. For the experiment described in Table II, 10 cc. of the horse serum were dialysed for 48 hours in an animal membrane ("Fischblase") against running tap-water. To avoid bacterial action some thymol crystals were added to the serum. During the dialysis the volume of the serum increased to 14 cc. The precipitated euglobulin was separated from the dialysed serum by centrifuging. This euglobulin was also tested for inhibiting power, but proved to be without effect. Korschun has made the same observation. Half of the dialysed serum was used for the experiment, mentioned in Table II, while the remaining part—7 cc.—was brought back into the membrane and dialysed for further 48 hours. The volume of this second part thereby increased to 10·5 cc. This serum was used for the experiment in Table III.

In Fig. 1 the inhibiting powers of the dialysed sera are graphically shown, and compared with the inhibiting power of normal horse serum at 37° (Table IV). Like all the curves in this paper, those in Fig. 1 indicate the effect of the inhibiting substance in question, expressed by the ratio between the maximum amount of rennet, neutralised by the inhibiting substance, and the minimal quantity of rennet (i.e. 0·0015 cc.), required for the coagulation of 10 cc. of the milk used, without any addition of serum.

TABLE II.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·14 cc. of a horse serum, dialysed for 48 hours	0·02 cc.	0·08 cc.	0·12 cc.	0·14 cc.	0·16 cc.	0·16 cc.	0·16 cc.	0·16 cc.	0·16 cc.

As seen above, 0·14 cc. horse serum dialysed for 48 hours is equal to 0·1 cc. of normal horse serum.

TABLE III.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·21 cc. horse serum, dialysed for 96 hours ..	0·015 cc.	·06 cc.	0·11 cc.	0·13 cc.	0·15 cc.	0·15 cc.	0·15 cc.	0·15 cc.	0·15 cc.

0·21 cc. of the horse serum dialysed for 96 hours corresponds to 0·1 cc. normal horse serum.

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The curves in Fig. 1 show perhaps more clearly than the corresponding tables that the period for which rennet and dialysed serum are kept before the milk is added to the complex is of decisive importance for the inhibiting power of these sera. The dialysis therefore does not differentiate between the true antibody and the pseudo-antirennet with the loss of the latter, but decreases

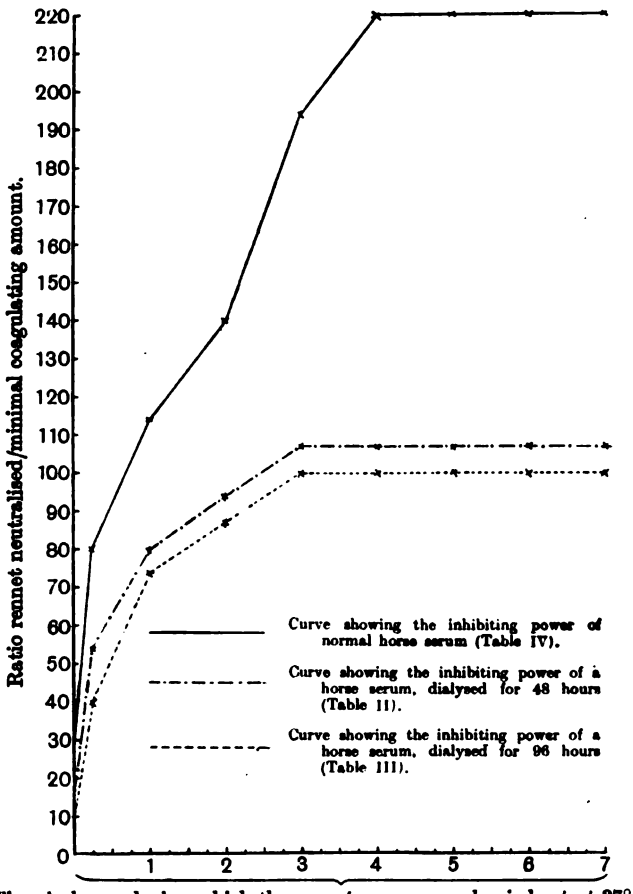

Fig. 1. Showing the inhibiting power at 37° of normal and dialysed horse serum.

the total inhibiting power of the serum, a decrease which is evidently greater the longer the dialysis lasts.

Even if the above data do not give any direct information as to the nature of the true antibody of horse serum, two conclusions may be drawn from them. Firstly, that this body cannot be a true antibody in the strict sense of the term, since the power of a true antibody is not decreased by dialysis

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of the serum in which it is contained[1]. And secondly, they show that there is no such contrast between "true antibody" and "pseudo-antirennet" in their relations to dialysis, as Korschun supposed, which to him was one of the essential arguments for regarding them as two different substances.

(b) *Pseudo-antirennet; its mode of action and its nature.*

When a normal horse serum acts on rennet at 37° its inhibiting effect is much greater than at 20°, as shown in the following table (see Fig. 1).

TABLE IV.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·1 cc. of normal horse serum	0·04 cc.	0·12 cc.	0·17 cc.	0·21 cc.	0·29 cc.	0·33 cc.	0·33 cc.	0·33 cc.	0·33 cc.

This observation also agrees with Hedin's statement that the inhibiting power of the serum increases up to a certain equilibrium proportionally, not only to the time, but also to the temperature at which the serum acts on the rennet. Korschun regards this increase in the inhibiting power as being due to the so-called pseudo-antirennet. As this body is supposed to be extremely thermostable, it ought to be separated from the thermolabile "true antibody" by heating the horse serum. An experiment made with a heated serum therefore should show the effect of the pseudo-antirennet better than the normal horse serum was able to do.

Following Korschun's instructions, an experiment like this is best made with a horse serum diluted in the proportions (1 + 5) with distilled water or tap-water, and then boiled for some time. In making the experiment, 10 cc. of the above-mentioned horse serum were therefore mixed with 50 cc. of distilled water, and the mixture kept in a boiling water-bath for one hour. The inhibiting effect of the serum dilution thus treated was determined in the usual way and found to have the following values.

[1] The only apparent cause for a decrease in the power of a true antibody would be that the dialysis had deprived the serum, in which the antibody is contained, of its content of NaCl. But even if NaCl be added to the dialysed horse serum the inhibiting power of the latter is not altered.

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TABLE V.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·6 cc. of a boiled horse serum dilution (1 + 5)	0·004 cc.	0·08 cc.	0·20 cc.	0·29 cc.	0·31 cc.	0·31 cc.	0·31 cc.	0·31 cc.	0·31 cc.

0·6 cc. of the boiled serum dilution contains 0·1 cc. horse serum.

The use of so large a quantity of serum dilution made the precipitation of the casein, formed in the samples by the action of the rennet, extremely difficult, as the dilution had lowered the concentration of the calcium salts in the milk too much. Normal conditions were, however easily brought about by adding some drops of a calcium chloride solution (4 %).

This experiment plainly shows that by heating the horse serum a considerable decrease is effected in the amount of rennet instantaneously neutralised by the true antibody. On the other hand, the heating does not appear to have changed the inhibiting power of the pseudo-antirennet, as the quantity of rennet neutralised at 37° by the heated serum practically agrees with what is destroyed at the same temperature by normal horse serum (Table IV). Notwithstanding this, it would be erroneous to conclude, as Korschun does, that the heating has destroyed the true antibody without hurting the pseudo-antirennet. A closer examination of the conditions, obtaining in the experiment to which Table V refers, shows that a horse serum, diluted (1 + 5) and heated to 70° for a quarter of an hour, has a higher inhibiting power at 37° than the boiled dilution and that an unheated dilution (1 + 5) has a still greater effect at the same temperature. By heating a serum dilution therefore we weaken its total inhibiting power, also that part of it which is due to the pseudo-antirennet. Tables VI and VII and Fig. 2 illustrate this.

TABLE VI.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·6 cc. of a horse serum dilution (1 + 5), heated to 70° for a quarter of an hour ..	0·006 cc.	0·09 cc.	0·21 cc.	0·29 cc.	0·32 cc.	0·35 cc.	0·38 cc.	0·38 cc.	0·38 cc.

0·6 cc. of the dilution used contained 0·1 cc. horse serum.

TABLE VII.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·6 cc. of a horse serum dilution (1 + 5)..	0·04 cc.	0·12 cc.	0·27 cc.	0·33 cc.	0·37 cc.	0·40 cc.	0·42 cc.	0·42 cc.	0·42 cc.

0·6 cc. of the dilution used contained 0·1 cc. horse serum.

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The heating has thus considerably damaged both the true antibody and the pseudo-antirennet, with the difference that the destruction of the first

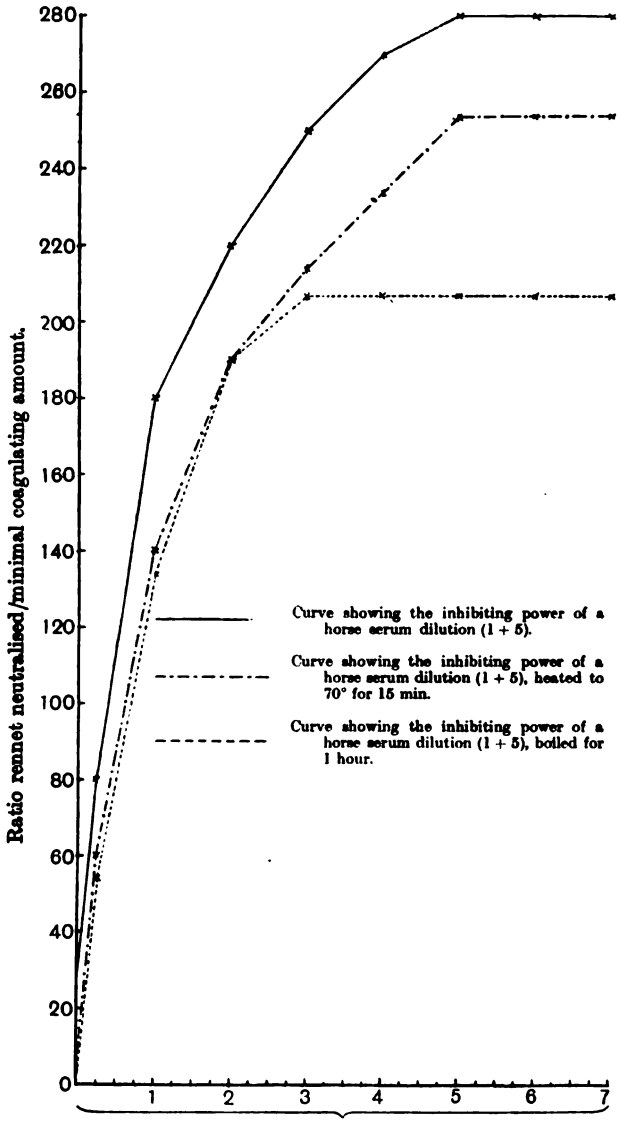

Fig. 2. Showing the inhibiting power at 37° of heated and unheated horse serum dilutions (1 + 5).

is, so to speak, complete, while the pseudo-antirennet is still active to a high degree and cannot be entirely destroyed. Then it must be assumed that the destruction of the pseudo-antirennet has practically come to an end, since a

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serum dilution, boiled for one hour, gives values for its inhibiting power which are but little less than those shown by a dilution heated for a quarter of an hour to 70°. This partial destruction of the pseudo-antirennet and the increase in the inhibiting power by diluting it would hardly be explainable if the pseudo-antirennet, as supposed by Korschun, were a substance present in normal horse serum as an independent body. On consideration the above-mentioned facts rather indicate that the term pseudo-antirennet, as applied to the inhibiting power of heated and unheated serum dilutions, covers at least three properties of normal horse serum, i.e. its faculty of regulating the [H'] in its dilutions, its alkalinity and its thermolability.

The importance of the first of these properties is apparent on an examination of the effect which dilution of normal horse serum has on its inhibiting power. Allusion has already been made to Hedin's statement that dilution of the normal serum does not change its inhibiting power; this is not in conformity with my own experiments. In view of the method employed by Hedin in his experiments, it is, however, impossible to attach importance to his deductions. By using Korschun's exact methods it is obvious that the inhibiting power is increased considerably by dilution of the serum. This may be seen from Tables VIII and IX, which concern two experiments made with diluted horse sera.

TABLE VIII.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·3 cc. of a horse serum diluted (1+2)..	0·04 cc.	0·12 cc.	0·25 cc.	0·28 cc.	0·31 cc.	0·36 cc.	0·36 cc.	0·36 cc.	0·36 cc.

0·3 cc. of the serum dilution used contained 0·1 cc. horse serum.

TABLE IX.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·6 cc. of a horse serum diluted (1+5)..	0·04 cc.	0·12 cc.	0·27 cc.	0·33 cc.	0·37 cc.	0·40 cc.	0·42 cc.	0·42 cc.	0·42 cc.

0·6 cc. of the serum dilution used contained 0·1 cc. horse serum.

This increase in the inhibiting power becomes more apparent on examination of the [H'] of the normal horse serum and its dilutions. Tested electrically the [H'] of the normal horse serum in question gives the value

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$p_{H^{\cdot}} = 7{\cdot}99$. Diluting the serum (1 + 5) its alkalinity does not decrease. On the contrary, measured at 37° its [H˙] gives values which vary from $p_{H^{\cdot}} = 8{\cdot}20$ to $p_{H^{\cdot}} = 7{\cdot}90$, depending on the time at which they are measured, either directly the dilution is made or later. The [H˙] of the dilution (1 + 2) agrees, as does that of the dilution (1 + 5), with the [H˙] of the normal horse serum.

Considering the extreme sensitiveness of rennet towards even very small quantities of alkali, it seems natural to connect the increase of the inhibiting power of the dilutions with the tendency of the serum to maintain normal [H˙] even in its higher dilutions. Indeed we know that the amount of rennet inhibited by an alkali solution is dependent on the [H˙] of this solution only, and not on the exact number of H- or OH-ions present. The same fact must therefore determine the action of the serum dilutions if their [H˙] has any influence on their increased inhibiting power. From this point of view it is, however, also justifiable to suppose that a greater quantity, either of the alkaline solution or of the serum dilutions, will destroy more rennet than a smaller amount is able to do. To explain this we may suppose that 0·1 cc. of alkaline solution of a certain [H˙] neutralises 0·02 cc. rennet solution; 0·6 cc. of the same alkaline solution should then neutralise $6 \times 0{\cdot}02$ cc. = 0·12 cc. rennet. That this occurs when the alkali is kept in six tubes each containing 0·1 cc. is obvious. If 0·6 cc. of the alkali is brought into one single tube, the same amount of rennet should be inhibited, as in this case also we have 0·02 cc. rennet corresponding to each of the alkali's six units of 0·1 cc. I have tried experimentally to ascertain how far this agrees with the actual, and found that 0·6 cc. of a Na_2HPO_4 solution of a certain [H˙] inhibits seven times more rennet than 0·1 cc. of the same phosphate solution. This result is very satisfactory, as experimental errors must always be considerable in work of this kind.

If a greater quantity of alkali is thus able to destroy more rennet than a smaller, the serum and its dilutions must do the same, as their [H˙] is also constant; and 0·6 cc. of a serum dilution (1 + 5) must therefore inhibit more rennet than 0·3 cc. serum dilution (1 + 2), and this 0·3 cc. again more than 0·1 cc. of normal horse serum. The question whether the increase in the inhibiting power of the serum can be due to its property of maintaining normal [H˙] in its dilutions therefore resolves itself into one, as to whether an increase in the inhibiting power, equal to that shown by the serum dilutions, can be caused by an alkaline solution of the same [H˙] as the serum—$p_{H^{\cdot}} = 7{\cdot}99$ (at 37°)—and used in the same quantities as this.

The increase in the inhibiting power of the serum dilution (1 + 2) is equal

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to 0·03, this value being the difference between the maximum amount of rennet (0·33 cc.) inhibited by 0·1 cc. of normal horse serum at 37° and the greatest quantity (0·36 cc.) destroyed at the same temperature by 0·3 cc. of the serum dilution (1 + 2).

The increase in the inhibiting power of the solution (1 + 5) is 0·06, as 0·3 cc. of the dilution (1 + 2) inhibits 0·36 cc. rennet, and 0·6 cc. of the dilution (1 + 5) inhibits 0·42 cc.

As regards the alkaline solution, it has already been pointed out that the increase in its inhibiting power is proportional to the amount of solution used. 0·3 cc. alkaline solution of a certain [H˙] therefore inhibits three times as much rennet as 0·1 cc. of the same solution. 0·1 cc. alkali of the concentration required[1] inhibits 0·04 cc. rennet, and 0·3 cc. of this alkali therefore ought to inhibit $3 \times 0{\cdot}04 = 0{\cdot}12$ cc. rennet, giving an increase in the inhibiting power equal to $0{\cdot}12 - 0{\cdot}04 = 0{\cdot}08$. On the other hand, 0·6 cc. of the alkali in question[2] will inhibit $6 \times 0{\cdot}04 = 0{\cdot}24$ cc. rennet, which represents an increase in the inhibiting power equal to $0{\cdot}24 - 0{\cdot}12 = 0{\cdot}12$. The experiments upon which these calculations are based are somewhat crude and the values cannot be regarded as absolutely exact. Nevertheless they suffice to show that an increase in the inhibiting power such as is shown by the serum dilutions is to be expected when they are used in increasing quantities.

With respect to the other properties which are necessary for the action of the pseudo-antirennet, the alkalinity and the thermolability of the serum, their importance is best shown by a closer examination of the inhibiting power of a boiled horse serum.

In view of the results of the above-mentioned experiments one might expect the reduction of the inhibiting power of the heated serum dilutions to be due to a decrease of their [H˙] caused by the treatment. This, however, was not the case, as the [H˙] of the boiled serum dilution gave roughly the same value as that shown by the unheated dilutions. Nevertheless there is an important relation between the inhibiting power of the boiled serum and its [H˙]. This became evident by a determination of the inhibiting power of 0·6 cc. of the phosphate solution at 37°. The experimental data are given in Table X.

[1] A solution of Na_2HPO_4 and KH_2PO_4 was used, made according to Sörensen's [1909] instructions by mixing 9·75 cc. $n/15$ Na_2HPO_4 and 0·25 cc. $n/15$ KH_2PO_4; tested at 18° this mixture has the $[H^{\cdot}]$ $p_{H^{\cdot}} = 8{\cdot}038$.

[2] In an experiment already mentioned 0·6 cc. phosphate solution neutralised 0·28 cc. rennet, and not 0·24 cc.

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TABLE X.

	Time during which the rennet-phosphate complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·6 cc. of a phosphate solution with the $[H^{\cdot}]p_{H^{\cdot}} = 8{\cdot}038$ (18°) ..	0·003 cc.	0·04 cc.	0·19 cc.	0·23 cc.	0·28 cc.	0·28 cc.	0·28 cc.	0·28 cc.	0·28 cc.

As can be seen from the curves in Fig. 3 there is a striking agreement between the inhibiting effects of the phosphate solution and the boiled serum

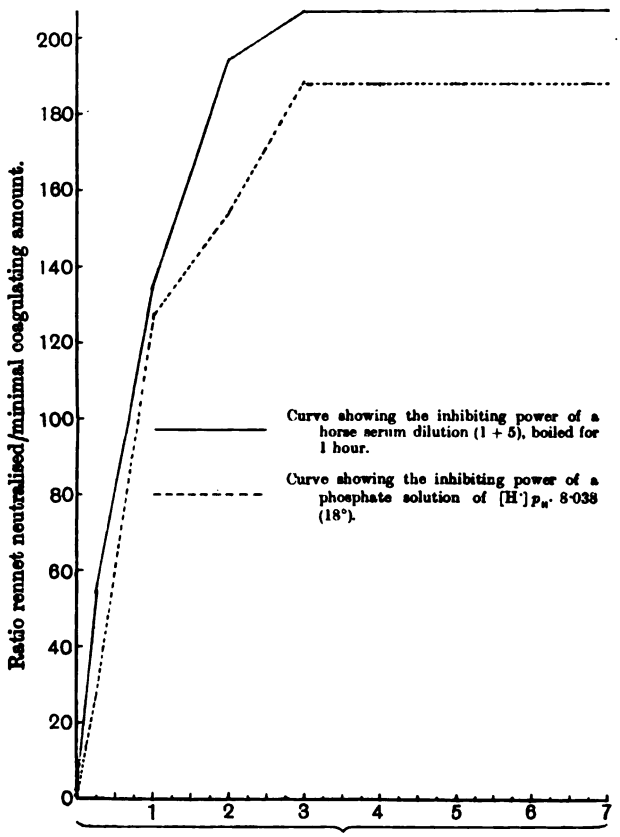

Fig. 3. Showing the inhibiting power at 37° of a boiled horse serum dilution (1 + 5) and of a phosphate solution of nearly the same [H·].

dilution. There appears no reason to doubt that this agreement is due to the fact that the inhibiting power of the boiled horse serum is an effect of its [H·] only. This will be sufficient to indicate the importance of the alkalinity

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of the serum with respect to its inhibiting power. It therefore only remains to be shown that the inhibiting power is dependent also on the thermolability of the serum.

The value of the [H·] is clearly as important for the inhibiting power of the serum dilutions as it was found to be for the action of the boiled serum. The inhibiting power of the dilution (1 + 5), for instance, must therefore be regarded as a mixed effect of its [H·] and of various other supplementary properties (substances?). In addition to the effect of the [H·] the inhibiting power of this dilution therefore also shows the effect of these properties. As can be seen from Table XI they also relate to the pseudo-antirennet, as their inhibiting power increases proportionally to the time the serum acts on the rennet.

TABLE XI.

	Time during which the serum or the phosphate solution acts on the rennet at 37°								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·6 cc. of a serum dilution (1 + 5)	0·04 cc.	0·12 cc.	0·27 cc.	0·33 cc.	0·40 cc.	0·42 cc.	0·42 cc.	0·42 cc.	0·42 cc.
The maximum amount of rennet neutralised by 0·6 cc. of a phosphate solution of the same [H·] as the horse serum	0·003 cc.	0·04 cc.	0·19 cc.	0·23 cc.	0·28 cc.	0·28 cc.	0·28 cc.	0·28 cc.	0·28 cc.
The inhibiting effect of the supplementary properties of the horse serum	0·037 cc.	0·08 cc.	0·08 cc.	0·10 cc.	0·12 cc.	0·14 cc.	0·14 cc.	0·14 cc.	0·14 cc.

Calculations similar to that made in Table XI show that the effect of these supplementary properties of the serum dilution (1 + 5) is greatly reduced by heating it to 70°. In a boiled dilution their effect has completely disappeared. As the thermolability of the serum is thus decisive for the effect of these properties, it must also be of importance for the action of the pseudo-antirennet, since the properties in question, as shown in Table XI, are part of the pseudo-antirennet.

Some interesting light as to the nature of these supplementary properties, or substances, of normal horse serum may be obtained from the experiments already discussed. As they cannot be demonstrated in a boiled horse serum dilution, their effect is obviously due to the proteins of the serum, or perhaps to their colloid qualities. Moreover, the effect of the properties in question increases proportionally to time, and to the temperature at which the serum acts on rennet. This can be seen from Tables I and XII. Table I showed the inhibiting power of 0·1 cc. of normal horse serum at 20°. But the figures

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at the same time also practically show the inhibiting effect of the supplementary properties at 20°, as the [H'] of 0·1 cc. of normal horse serum is hardly of any importance for the amount of rennet destroyed at this temperature. Experiments showed that 0·1 cc. of the phosphate solution neutralised 0·003 cc. rennet at 20°, an amount which does not increase even if the solution acts on rennet for several hours.

The inhibiting power of the supplementary properties at 37° is shown in Table XII.

TABLE XII.

	Time during which the serum or the phosphate solution acts on the rennet at 37°								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·1 cc. of normal horse serum	0·04 cc.	0·12 cc.	0·17 cc.	0·21 cc.	0·29 cc.	0·33 cc.	0·33 cc.	0·33 cc.	0·33 cc.
The maximum amount of rennet neutralised by 0·1 cc. phosphate solution of the same [H'] as the horse serum	0·003 cc.	0·02 cc.	0·04 cc.	0·04 cc.	0·04 cc.	0·04 cc.	0·04 cc.	0·04 cc.	0·04 cc.
The effect of the supplementary properties of the horse serum is therefore equal to	0·037 cc.	0·10 cc.	0·13 cc.	0·17 cc.	0·25 cc.	0·29 cc.	0·29 cc.	0·29 cc.	0·29 cc.

The effect of these properties is thus considerably increased by raising the temperature at which the experiment is made.

Furthermore, some light is thrown on them from Table XII. Compared with the figures in Table XI, which showed their inhibiting power at high dilution, the figures in Table XII indicate that their effect is greater when they act on rennet in concentrated than in diluted form.

The real cause of the appearance of these properties, or substances, cannot be determined from the experiments discussed above. Korschun's "true antibody" is certainly either identical with them or part of them. Nevertheless the use of this expression for the explanation of the action of the normal serum on rennet is not to be recommended. This action cannot be due to a true antibody, but is more probably attributable to physico-chemical effects. On the other hand, Hedin's conclusion that this action is a mere adsorption must undoubtedly only be a partial explanation.

II. The Inhibiting Effect of Normal Rabbit Serum on Rennet.

As can be seen from the following table the action of normal rabbit serum on rennet has the same characteristics as that of normal horse serum, with the difference merely that its effect is distinctly weaker.

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TABLE XIII.

No.	Material	Temp.	Vol. of serum	Maximum amount (in cc.) of rennet neutralised when the rennet-serum complex is kept for the following times before milk is added to it								
				0	0·25	1	2	3	4	5	6	7 hours
1	Normal rabbit serum	20°	0·1 cc.	0·004	0·008	0·01	0·015	0·03	0·03	0·03	0·03	0·03
2	Normal rabbit serum	37°	0·1 cc.	0·004	0·025	0·085	0·11	0·14	0·17	0·17	0·17	0·17
3	Normal rabbit serum dialysed for 48 hours through an animal membrane	37°	0·35 cc. of the dialysed serum (=0·1 cc. undialysed serum)	0·0015	0·02	0·07	0·09	0·15	0·17	0·17	0·17	0·17
4	Rabbit serum diluted (1+5)	37°	0·6 cc. diluted serum (=0·1 cc. normal serum)	0·004	0·025	0·09	0·13	0·16	0·18	0·21	0·21	0·21
5	Rabbit serum diluted (1+5) and heated to 70° for 0·25 hour	37°	0·6 cc. diluted serum (=0·1 cc. undiluted serum)	0·0015	0·006	0·12	0·15	0·15	0·15	0·15	0·15	0·15

The experiments from which these numbers are derived were arrived at in the same way as those dealing with the inhibiting power of normal horse serum. The same rabbit serum was used for all experiments. In addition two other rabbit sera were tested, one of which showed a lower inhibiting power than that used for the experiments quoted. The intensity of the inhibiting power thus varies not only with different species of animals, but also with individuals of the same species, a fact which has already been recognised.

III. The Inhibiting Power of a Rennet Antiserum.

The question of the possible identity of artificial antirennet and that found in normal horse serum has already been referred to in the introduction. Previous researches, Korschun's and Morgenroth's [1900] especially, appeared to point in this direction, at any rate as far as those rennet antisera were concerned, which were made by immunising animals, e.g. goats or rabbits, with a true rennet enzyme. The antirennet of the normal serum, or at least a part of it, was regarded as a true antibody, formed in the serum of the animal in question by autoimmunisation with rennet, originating from the mucous membranes of its stomach. As the results of the experiments mentioned in

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this paper affect this conception of the inhibiting effect of normal horse serum in various respects, it appeared of interest to ascertain whether the action of the rennet antiserum could be explained in the same manner as that of the normal serum, or whether in this antiserum there occurred a typical antibody with properties similar to those claimed by Korschun for the antibody of normal horse serum.

The rennet antiserum, employed in the experiments discussed below, was obtained in the following manner.

The immunisation was accomplished in The Swiss Institute for Dairy Bacteriology, Liebefeld near Berne. As antigen a pure rennet powder (Merck) with the coagulating power $1-1{\cdot}5 \times 10^6$ was used. The animals were injected with an extract of this rennet powder in the strength of 1·0 g. of rennet powder to 10 cc. of sterile distilled water. For each injection, given every other day, a fresh quantity of extract was made. At the beginning of the treatment the rabbits used for the immunisation did not show any loss of weight, but later on, after the seventh injection, a rather heavy fall could be noticed. Two of the rabbits died at the end of the treatment with typical anaphylactic symptoms. The subcutaneous injection caused a somewhat considerable local infiltration. Contrary to what was expected, the rennet antiserum was found to be very durable. Even at the end of a year its inhibiting power was entirely unimpaired.

Unfortunately it has been necessary to limit the number of experiments originally planned with this antiserum, as the quantity available was altogether inadequate, especially as each experiment had to be carried out two or three times.

It is interesting to note that the inhibiting power of the rennet antiserum at 20° and 37° agrees well with the properties claimed by Korschun for a serum containing a true antirennet. See Table XIV.

TABLE XIV.

The inhibiting power of rennet antiserum at 20° and 37°.

	Time during which the rennet-serum complex is kept before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
1. The maximum amount of rennet neutralised by 0·1 cc. of a rennet antiserum, at 20°	0·03 cc.	0·03 cc.	0·03 cc.	0·03 cc.	0·03 cc.	0·03 cc.	0·03 cc.	0·03 cc.	0·03 cc.
2. The maximum amount of rennet neutralised by 0·1 cc. of a rennet antiserum, at 37°	0·03 cc.	0·045 cc.	0·065 cc.	0·09 cc.	0·115 cc.	0·13 cc.	0·13 cc.	0·13 cc.	0·13 cc.

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At 20° the antiserum thus neutralises a considerable quantity of rennet, which does not increase even if the rennet-antiserum complex is kept for seven hours at 20°. Furthermore, the effect of the true antirennet, i.e. the capacity of the antiserum for destroying a maximum amount of rennet in a very short time (a few seconds), has considerably increased owing to the immunisation, whereas the effect of the pseudo-antirennet at 37° is still unaltered, as the difference in the inhibiting power between the pseudo-antirennet of the normal rabbit serum and that of the antiserum (Tables XIII, No. 2, and XIV, No. 2) is no doubt due to individual differences in the inhibiting power of sera of the same species, and not to a decrease in the effect of the pseudo-antirennet owing to the dialysis.

At this point, however, the agreement between the effect of the antiserum and the properties claimed by Korschun for the true antirennet ceases. Testing the effect of the antiserum under other conditions, the agreement rather seems to have been accidental. Dialysis, e.g., causes a decrease in the entire inhibiting power of the antiserum (Table XV), just as was the case with the dialysed horse serum.

TABLE XV.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·22 cc. of a rennet antiserum dialysed for 48 hours	0·01 cc.	0·03 cc.	0·05 cc.	0·08 cc.	0·08 cc.	0·08 cc.	0·08 cc.	0·08 cc.	0·08 cc.

3 cc. of antiserum were dialysed for 48 hours, its volume thereby increasing to 6·5 cc. 0·22 cc. of the dialysed antiserum therefore is equal to 0·1 cc. untreated antiserum.

This decrease in the inhibiting power of the antiserum is not consistent with the presence of a true antibody in this serum.

Similarly, it is noteworthy that the effect of the pseudo-antirennet is increased far more by diluting the antiserum (Table XVI) than by diluting normal rabbit serum (Table XIII, No. 4). This would hardly be the case if the pseudo-antirennet, as assumed above, had not been altered by the immunisation.

TABLE XVI.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0·6 cc. of a rennet antiserum diluted (1 + 5)	0·01 cc.	0·06 cc.	0·20 cc.	0·22 cc.	0·23 cc.	0·24 cc.	0·24 cc.	0·24 cc.	0·24 cc.

0·6 cc. of the diluted antiserum contained 0·1 cc. antiserum.

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The increase in the effect of the pseudo-antirennet is more explicable if viewed from the same standpoint as the changes in the inhibiting power of normal horse serum caused by dilution. Unfortunately, the experiments made with the antiserum had not left a sufficient quantity for a determination of its [H·]. Using Sörensen's indicator method it was, however, found that the antiserum was more alkaline than normal rabbit serum, but less alkaline than normal horse serum[1]. Dilutions of the antiserum therefore have a smaller [H·], i.e. a higher alkalinity, than dilutions of normal rabbit serum, as the sera are able to maintain normal [H·] even in high dilutions. As a matter of course the dilutions of the antiserum therefore must destroy more rennet than dilutions of normal rabbit serum.

There is another peculiarity in the inhibiting effect of the antiserum at 20°, which is not so easily explained as the changes in the action of the pseudo-antirennet. As can be seen from Tables XIII, No. 1, and XIV, No. 1, the maximum amount of rennet neutralised by normal rabbit serum at 20° is equal to that destroyed by the antiserum at the same temperature. The velocity of reaction of the destruction is, however, very different for the two sera, the antiserum neutralising the maximum amount in a very short time, normal rabbit serum, on the contrary, in four hours only. As regards the diluted antiserum, the velocity of reaction is less than that of the antiserum itself (Table XVI).

Changes in the velocity of reaction, such as are shown by the antiserum, are well known from the study of the action of adsorbing bodies [Bayliss, 1906]. As was the case for the antibody, these changes are proportional to the concentration of the adsorbing body; adsorption increasing with the concentration. The peculiarity of the inhibiting effect referred to above is therefore possibly due to an increase in the concentration of some substance in the serum during the immunisation.

The researches on the inhibiting power of the antiserum here discussed do not favour the belief that the action of the rennet antiserum is of a special nature. On the contrary, they appear to indicate that this action cannot be due to the presence of a true antibody formed in the serum during immunisation.

[1] This is remarkable, as in his researches on the [H·] of the normal serum Michaelis [1914] points out that changes in the normal [H·] of the serum are extremely infrequent, at least as far as an increase is concerned.

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Conclusions.

1. The inhibiting effect of normal horse serum on the coagulating power of the rennet enzyme is not, as supposed by Korschun, due to two inhibiting substances, the so-called true antibody and the pseudo-antirennet. Neither can Hedin's explanation of this effect as a mere adsorption be regarded as fully satisfactory.

2. It can be shown furthermore that the [H˙] of normal horse serum is highly important for its inhibiting power.

3. Normal rabbit serum shows the same characteristics in its effect on rennet as normal horse serum.

4. The inhibiting power of a rennet antiserum made by immunising rabbits with rennet enzyme can hardly be due to the presence of a true antibody, as this serum also tends to show the characteristics of normal horse serum.

The author takes the opportunity of expressing his thanks to Prof. R. Burri, head of The Swiss Institute for Dairy Bacteriology, Liebefeld near Berne, who placed the standardised rennet solution at his disposal. He is also very indebted to Dr E. Atkin, of the Lister Institute, for the determinations of [H˙] mentioned in this paper.

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XIII. COUNTER DIFFUSION IN AQUEOUS SOLUTION.

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In the introduction to a description of some experiments published in this Journal W. A. Osborne and Miss L. C. Jackson [1914] have written: "If, for instance, there are two solutions one vertically placed above the other and in contact over a relatively small surface, and if both contain decinormal sodium chloride and one of them, necessarily the lower, contains ammonium sulphate of three molecular strength, then after some days' diffusion it is found that the sodium chloride concentration in the upper fluid is greater than decinormality whilst that in the lower fluid is correspondingly reduced."

In describing their analytical results the authors show that these conclusions were based solely on Cl and SO_4 determinations and that it was assumed that Cl represented NaCl and SO_4 represented $(NH_4)_2SO_4$.

Realising that incorrectness in this assumption might permit the establishment of these observations as interesting extensions of already recognised diffusion phenomena, I repeated this experiment, supplementing the Cl and SO_4 determinations by those of K and (NH_4). As a matter of convenience KCl was used instead of NaCl. The results, given below, confirm in every respect Osborne and Jackson's analytical determinations, but the additional data with regard to K and (NH_4) lead me to suggest the following alterations in the conclusions drawn: "...then after some days' diffusion it is found that the ammonia has diffused into the upper fluid faster than the equivalent quantity of sulphate, with the result that chloride has increased in concentration in the upper fluid while sodium (potassium) has increased in concentration in the lower fluid."

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The experiments described in this note were chosen with the view of establishing the phenomenon as one resulting from the varying velocities of ionic diffusion.

Experimental.

To simplify explanation it is convenient to refer to the originally evenly distributed solute as the *test substance*, and the solute added to one solution only as the *diffusing substance*; also, in the case of simple inorganic electrolytes to speak of them as if they were completely dissociated.

The method of experiment devised by Osborne and Jackson was followed very closely. The volume of fluid above the tap was, as in their case, equal to that of the bore of the tap plus that below the tap. This was 15·75 cc. in some experiments and 17·5 cc. in others. Osborne used larger volumes in his experiments, an arrangement which demands longer diffusion periods for the same ionic differentiation of the test substance, but permits, because of the larger volumes of material provided, more accurate analytical determinations in the fluids after diffusion.

The bottom fluid is analysed before diffusion as a check on the analytical methods. After diffusion each was well mixed and analysed in the same manner.

Experiment 1.

Test substance 0·15 *N KCl*; *diffusing substance* 0·790 *N* HNO_3.

Volume of top fluid = volume of bottom fluid = 15·75 cc.

		K	Cl	H	NO_3
Before diffusion					
	Top fluid	0·1508 N	0·1508 N	—	—
	Bottom fluid	0·1508 N	0·1508 N	0·790 N	[0·790 N]
After diffusion for nine days					
	Top fluid	0·1388 N	0·1625 N	0·1250 N	[0·1013 N]
	Bottom fluid	0·1627 N	0·1398 N	0·6610 N	[0·6839 N]

The K was weighed as K_2SO_4; Cl weighed as AgCl; H by titration; NO_3 by difference, for the sum of the normalities of the anions and kations in any solution must be equal.

The figures above show that the H and NO_3 originally to be found only in the bottom fluid have diffused upwards into the top fluid but with unequal velocities. The H has moved faster than the NO_3. At the same time it is seen that the K and Cl originally both of them 0·1508 N throughout the whole system have diffused in such a fashion that K has increased in concentration in the lower fluid by 0·0119 N and Cl has increased in concentration in the upper fluid by almost exactly the same amount.

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Experiment 2.

Test substance 0·15 *N KCl*; *diffusing substance* 0·25 *N* $Ba(OH)_2$.

Volume of top fluid = volume of bottom fluid = 15·75 cc.

		K	Cl	Ba	OH
Before diffusion					
	Top fluid	0·1508 N	0·1508 N	—	—
	Bottom fluid	0·1508 N	0·1508 N	0·2525 N	0·2525 N
After diffusion for 14 days					
	Top fluid	0·1603 N	0·1413 N	0·0238 N	0·0380 N
	Bottom fluid	0·1413 N	0·1591 N	0·2244 N	0·2060 N

Ba was weighed as $BaSO_4$ and K weighed as K_2SO_4 in filtrate. A correction was made for solubility of $BaSO_4$. Cl weighed as AgCl, OH by titration.

Here Ba and OH have diffused from the lower fluid which alone originally contained them into the upper fluid. The OH has moved the faster. With regard to the test substance KCl the K has increased in concentration by 0·0095 N in the top fluid and the Cl has increased by 0·0091 N, nearly the same amount, in the lower fluid.

Experiment 3.

Test substance 0·15 *N KCl*; *diffusing substance* 0·773 *N* NH_4OH.

Volume of top fluid = volume of bottom fluid = 17·5 cc.

		K	Cl	NH_4	OH
Before diffusion					
	Top fluid	0·1508 N	0·1508 N	0·7730 N	0·7730 N
	Bottom fluid	0·1508 N	0·1508 N	—	—
After diffusion for six days					
	Top fluid	0·1512 N	0·1500 N	0·7007 N	0·6971 N
	Bottom fluid	0·1521 N	0·1508 N	0·0723 N	0·0760 N

Here because of its lower specific gravity the solution containing the diffusing substance had to be placed on top. Although of three times the normality of the baryta used as diffusing substance the effect in upsetting the even distribution of K and Cl is so small as to be indistinguishable in the face of experimental error.

Experiment 4.

Test substance 0·15 *N KCl*; *diffusing substance* 3 *N* $(NH_4)_2SO_4$.

Volume of top fluid = volume of bottom fluid = 17·5 cc.

		K	Cl	NH_4	SO_4
Before diffusion					
	Top fluid	0·1508 N	0·1508 N	—	—
	Bottom fluid	0·1508 N	0·1508 N	2·91 N	2·91 N
After diffusion for 15 days					
	Top fluid	0·1394 N	0·1581 N	0·3365 N	0·2609 N
	Bottom fluid	0·1577 N	0·1434 N	2·6100 N	2·5933 N

NH_4 by Kjeldahl; SO_4 weighed as $BaSO_4$; Cl weighed as AgCl. The determination of such small quantities of K in the presence of so much ammonium sulphate could not be performed with accuracy; in driving off the $(NH_4)_2SO_4$ so that the K_2SO_4 remaining might be weighed a loss of two or three per cent. seemed unavoidable. This fact is reflected in the inconsistencies in the analytical figures for the fluids after diffusion.

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The analytical figures of these four experiments, together with those of Osborne and Jackson, suggest the following generalisation with regard to diffusion experiments conducted in this fashion.

The ion of the test substance having the opposite sign to the faster ion of the diffusing substance accumulates in the direction of the diffusion; the other ion of the test substance accumulates in the other direction. When the diffusing substance is feebly ionised such change in the distribution in the ions of the test substance does not occur.

Further remarks in this note will, for this reason, refer only to Experiments 1, 2 and 4 above.

Theoretical.

In all solutions it is a necessary condition that at any part of the volume positive and negative ions must be present in amounts electrically compensating. Those solutions in which some process of natural diffusion is occurring or has occurred are, in view of the fact that they manifest no external electrical phenomena, no exception to this rule. When, therefore, an electrolyte diffuses into an unionisable solvent its two ions must diffuse in company whether they are dissociated or free. If they differ in intrinsic mobility the faster ion will tend to diffuse ahead of the other, but because of the intense electrical field so established will succeed in doing so only to an inappreciable degree. By breaking down this field, however, the process may be allowed to proceed. If this is done by the direct application of an electric field from external sources the process is *electrolysis*. If it is the result of a differential migration of the ions of another electrolyte introduced into the system whereby all potential gradients are neutralised it is *counter diffusion*.

At this point it will be well to distinguish between what may be deduced from first principles with regard to these experiments and what facts are learnt from the analytical results.

In experiments arranged in this manner, with the top and bottom fluids exactly equal in volume, the test substance equally distributed throughout, and the diffusing substance originally in the bottom fluid only, fundamental space relationships and the principle of electrical compensation demand:

(*a*) The sum of the normalities of the anions is equal to the sum of the normalities of the kations in each fluid both before and after the experiment. [This is true, of course, of every solution.]

(*b*) The change of normality of either ion of the test substance in the

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top fluid, owing to diffusion, is equal and opposite in sign to the change of normality of the same ion in the bottom fluid.

(*c*) The sum of the changes of normality of the ions of the test substance in either fluid is equal but opposite in sign to the sum of the changes of normality of the ions of the diffusing substance in that fluid, if normalities of negatively charged ions are treated as negative quantities and the summation performed algebraically.

These three generalisations, deduced directly from first principles, provide an excellent check on analytical results. They may be represented in the general case by a set of simple simultaneous equations insufficient in number to determine absolutely the values of the unknown quantities.

The *experiments* made provide *further* information:

(α) That ion of the diffusing substance which is usually regarded as having the greater intrinsic velocity diffuses upwards to an extent greater than does the other; at least it does so in the three experiments above where the ions of the test substance, KCl, have approximately equal intrinsic velocities.

(β) The ratio of the nett migrations of the two ions of the diffusing substance is much less than the ratio of their accepted intrinsic velocities.

(γ) The migrations of K and Cl past the plane of the original boundary separating the two fluids are approximately equal; presumably because of their nearly equal migration velocities. These ions therefore share equally in the task of compensating for the unequal migrations of the ions of the diffusing substance.

It may be noted that it follows from the generalisations (*c*) and (β) above, that the nett migrations of the ions of the test substance past the plane of the original boundary must be smaller than the nett migrations of the ions of the diffusing substance.

Examples of all these generalisations may be seen by referring to the figures of Experiment 1 and those deduced therefrom given below:

	Test substance		Diffusing substance	
	K +	Cl −	H +	NO_3 −
Change of normality of top fluid, i.e. nett migration past the plane of the original boundary	−0·0120	+0·0114	+0·1250	+0·1013
	(sum = −0·0234)		(sum = +0·0237)	

It follows, too, that when a binary electrolyte diffuses into water it becomes, to a minute extent, acid or alkaline according as the anion or kation of the diffusing substance is the faster. By adding litmus to

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the water ahead of the diffusion front Durrant [1906] has observed the development of alkali in this manner by the diffusion of nitric acid.

Beyond those of Osborne and Jackson, who originated this method, I have not been able to find any descriptions of experiments where *differential* diffusion has been followed analytically. Durrant's observations [1906], although quantitative, were made rather on the distance migrated by different ions in the same time, and not upon the differences in quantity of the different ions passing a certain point.

The main interest in these results, however, lies in their relation to the theory of boundary potentials, for they represent a summation of the diffusions between the two liquids and, therefore, an integration of a function of the contact potential. The recognised complexity of these calculations will be reflected in any further attempt to predict from accepted data the results obtained above by analysis.

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XIV. THE DIGESTION AND ABSORPTION OF PROTEIN AND FAT IN NORMAL AND DEPANCREATISED ANIMALS.

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Von Noorden [1893] and Rubner [1879] showed that on a meat or concentrated protein diet, the protein N was well absorbed, the utilisation figure varying from 90 to 98 per cent. These and several other workers have shown that within limits the greater the amount of protein given in a digestible form the better is its utilisation.

Within the limits of normal dietary there are several factors which influence the absorption of fat. Von Noorden's experiments [1907, 1] with butter fat given in varying amounts in an otherwise uniform diet show clearly that proportionately more fat is excreted when the food is almost fat free than when a great deal of fat is administered. With 4·2 g. fat in the food the utilisation was 42·9 per cent., while with 80·2 g. the figure rose to 93·6 per cent.

The physical consistency and the melting point of the fat are of importance as has been demonstrated by Munk [1884] and by Arnschink [1890], who have fully worked out the relation of the melting point to facility of absorption. Again, as Bucheim [1874] pointed out with regard to the ready utilisation of cod liver oil, the percentage of free fatty acid present in a fat exercises a marked influence. Blumfeld and Hauser [1888] obtained quite as good results with butter and milk fat as with preparations containing a certain percentage of free fatty acid.

As far back as 1851, Traube and others showed that it was possible in

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diabetics to obtain a good utilisation of the food given. Hirschfeld [1891] in a paper on a new clinical form of diabetes stated that he could only recover 32 per cent. of the N ingested, while Brugsch [1906] found a loss of 20–25 per cent. of N and 50 to 60 per cent. of the fat ingested.

From a clinical point of view a considerable amount of work has centred around the question of the utilisation of fat in diabetes. E. Zunz and Mayer [1904] confirmed the statement that the closing of the pancreatic duct leads to a slowly ensuing disturbance of absorption. This impairment of function is slight compared with the disturbance following total extirpation with a diet containing no pancreas. In dogs, after total extirpation of the pancreas Abelmann [1890] found the following fat absorption figures: in small intestine non-emulsified fat, 32 per cent.; emulsified and milk fat, 80 per cent.; in the faeces on a milk diet there was a loss of 43–70 per cent. Müller [1887] and von Noorden [1907, 2] and others state that with disorders of pancreatic secretion it is the fat-splitting process which is affected. This view was opposed by Deucher, who obtained in severe disorders of the pancreas as complete a splitting of the fat as in the healthy organism, 62–80 per cent.

In the experiments described in this paper I have endeavoured to determine the amount of absorption which takes place in normal animals under a low and high protein and fat diet respectively, and to note what variation from the normal figures results when the animal is

(1) partially depancreatised (*a*) with subcutaneous graft of pancreas,
(*b*) with small piece of pancreas *in situ*,

(2) totally depancreatised, and

(3) under these conditions to see what effect is produced upon absorption by the addition of raw pancreas to the diet.

Methods of fat extraction and estimation.

The total quantity of faeces is dried in an air oven at room temperature, the process occupying 12–20 hours. The dried faeces are powdered thoroughly till the whole sample readily passes through a fine sieve, after which the sample is again rubbed up in a mortar till the whole is reduced to a very fine powder, which is then weighed. One gram is accurately weighed out and transferred to a Jena flask, 20 cc. 20 per cent. NaOH added, and the whole heated in a water-bath for three hours as recommended by Kumagawa and Suto [1908]. The warm alkaline solution is then poured into a separating funnel and acidified with HCl; the acid is added in small quantities and after

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each addition the separating funnel is well shaken under cold running water. When the whole is thoroughly cooled, about 50 cc. of ether are added and the mixture shaken at short intervals for a quarter of an hour and set aside for a few minutes to allow the solutions to stratify. This shaking is repeated three times and the separating funnel is then set aside for an hour so that all the ether may rise to the surface and a distinct thin film of demarcation between the two fluids become apparent. The lower layer is then separated, leaving the precipitate in the funnel, and the ether poured into a 200 cc. flask. The precipitate is dissolved in NaOH and shaken with ether; the separated acid layer is returned to the funnel and the whole extracted with ether twice. The ether is then evaporated, the residue redissolved in anhydrous ether and filtered through a capillary filter similar to that described by Mottram [1909]. The resulting solution is again evaporated, the residue dissolved in petroleum ether, allowed to stand for half an hour, and filtered. The petroleum ether is evaporated and the residue dried at 70° for 4–5 hours till of constant weight.

Folin and Wentworth [1910] have shown that everything taken out by organic solvents is not fat, and that it is impossible to determine separately free fatty acids and soaps. The ordinary fatty acids are very weak, and the extent to which they combine with bases to form soaps in faeces is not important. The equilibrium between soaps and fatty acids is influenced by so many factors, H_2S, CO_2, NH_4, heat, etc., during the drying, that the proportion of soaps, free fatty acids and neutral fat at the end of the process bears no relation to that existing at the time of defaecation. The new method described by Folin and Wentworth for the estimation of the fatty acids has been used. The solvent used was warm benzene and the dissolved fatty acid was titrated with N/10 alcoholic soda, phenolphthalein being used as an indicator. On several occasions the mean molecular weight of the fat of the faeces was determined, both with normal and diabetic samples, and under normal conditions of intestinal activity, and it was found to approximate closely to the molecular weight of stearic acid.

The estimation included the total fat, extracted as fatty acid plus cholesterol, lecithin and unsaponifiable substances present in the stool. The fatty acid was determined by the titration described above, while the difference between the fatty acid and the total fat extracted by petroleum ether was taken as an indication of the amount of lecithin and unsaponifiable substances in the faeces.

The N was determined by the Kjeldahl method.

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Animals.

The faeces for the periods were demarcated by an addition of bones to the diet. The dogs were weighed at regular intervals, the diabetic animals being weighed every day. While under observation the dogs were kept in metabolism cages. They were fed usually once a day (about 5 p.m.), no food being left in the cages over night. The food was weighed and precautions taken against wasting of the weighed amount.

Samples of the food were analysed from time to time. The following table gives the percentage analysis:

	N	Fat
Horse meat (cooked).. ..	4·1 to 4·41	3·0
Ox pancreas	2·89	12·1
Caseinogen	15·0	—
Puppy biscuit	3·14	3·48
Suet	0·730	82·0
Milk	0·52	3·5
Ereptone	12·8	—

Operations.

All the operations were performed by Professor Starling. The dogs were given a small dose of morphia hypodermically about two hours previously and then the operation was carried out under CE anaesthesia.

In the case of Dog I where a graft of the gland was left, all the pancreatic tissue was removed from the abdominal cavity, a small portion of the tail of the gland with its blood supply intact through a pedicle of mesentery being placed under the skin of the abdominal wall. The graft was functional, secreting a clear fluid for a short period after the operation, which could slowly digest fibrin.

With regard to Dog II, which was also partially depancreatised, a small portion of the gland was left *in situ* close to the main pancreatic duct, which was ligated peripherally. After removal of this piece of pancreatic tissue, the dog, which had a reduced sugar tolerance, became markedly diabetic, but remained in good condition for some time.

The Utilisation of Protein N and Fat in Normal Dogs.

Table I gives the total and percentage excretion of N and fat and the percentage of fatty acid of total petroleum ether extract, which latter includes lecithin, cholesterol and unsaponifiable substances, etc.

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TABLE I.

Normal Dogs.

Date		Dry wt. of Faeces g.	Total N	% N	Total fat	% fat	Total fatty acid	% fatty acid of total P. E. Extract
			1. Diet: puppy biscuit, 200 g.—5 days.					
Dec.	1	15	1·08	4·73	0·720	3·60	—	—
	3	20	0·870	4·62	1·038	7·42	—	—
			Weight, 10·15 kilos.					
			2. Diet: meat, 250 g.—5 days.					
	10	15	0·693	4·48	2·01	13·04	1·260	60
	11	20	0·574	2·87	1·59	7·98	0·963	60·5
	12	14	0·300	2·10	0·670	4·78	0·371	56·7
	14	16	0·493	3·08	0·592	3·20	0·273	53·6
			Weight, 9·70 to 9·65 kilos.					
			3. Diet: milk, 200 c.c.; puppy biscuit, 100 g.; meat, 100 g.—9 days.					
	18	17	1·05	6·23	1·67	9·80	0·91	54
	19	20	1·26	6·30	1·432	7·40	0·624	43·6
	20	23	1·16	5·04	1·45	7·80	0·759	52·1
	22	15·5	0·90	5·46	1·55	10·0	0·924	59·6
	24	24	1·377	5·74	1·69	7·0	0·824	48·8
			Weight, 10 to 9·6 kilos.					
			4. Diet: milk, c.c. per diem, 700, 700, 650, 600, 350, 250, 400, 400, 400. Puppy biscuit, 89 g. per diem (7th and 8th); 90 g. for last 3 days (9th, 10th and 11th).					
Jan.	6	23	1·03	4·48	6·716	29·2	4·301	64·2
	8	18·4	0·773	4·06	1·884	10·24	0·889	50·2
	9	19·5	1·133	5·74	1·840	9·46	0·866	47·8
	12	30	1·470	4·76	1·530	5·14	0·766	56·8
			Weight, 10 to 10·1 kilos.					
			5. Diet: milk, 400 c.c.; puppy biscuit, 90 g.; lemco, 4, 8, 12, 16, 30, 16, 16, 8 g. per day.					
Feb.	15	37·5	1·84	4·9	1·77	5·55	1·065	51·4
	17	22·5	1·230	5·45	1·07	4·35	0·639	65·1
	20	42·0	2·226	5·32	2·788	6·64	0·984	40·0
			Weight, 8·85 to 8·80 kilos.					
			6. Diet: meat, 400 g. first 2 days; 750 g. next 3 days; ereptone, 134·5 g.					
Mar.	4	33·3	1·84	5·53	2·84	8·86	1·230	45·0
	7	32·0	2·24	7·28	3·2	9·92	1·635	51·0
	9	20·6	1·241	6·02	2·04	9·90	1·179	54·5
			Weight, 9·10 to 9·00 kilos.					
			7. Diet: meat, 143 g.; suet, 72 g. per diem (average)—13 days.					
Jan.	18	27·2	1·104	4·06	3·56	13·16	2·086	58·3
	22	26·0	1·40	5·67	8·03	30·8	5·096	63·4
	25	20·0	1·176	5·88	5·08	28·8	3·692	64·0
	30	26·0	1·456	5·60	6·55	25·2	3·640	57·0
			Weight, 9·9 to 9·00 kilos.					

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TABLE I—*Continued*

Date		Dry wt. of Faeces g.	Total N	% N	Total fat	% fat	Total fatty acid	% fatty acid of total P. E. Extract
		8. Diet: caseinogen, 60 g.; palmine, 30 g. per diem for 7 days.						
Mar.	11	16·5	0·880	5·32	1·73	10·1	1·218	73·1
	13	12·1	0·545	4·55	2·34	19·3	1·224	52·8
	14	10·3	0·462	4·48	1·09	10·56	0·789	74·0
	15	11·0	0·531	4·83	0·891	8·10	0·625	70·2
	16	10·65	0·522	4·90	0·880	8·12	0·667	77·7
	18	21·6	0·937	4·34	2·268	11·08	1·944	86·0
		Weight, 8·90 to 8·75 kilos.						
		9. Diet: caseinogen, 60 g.; cod liver oil, 40 c.c. (17th–26th).						
Mar.	19	11·6	0·544	4·90	0·673	6·06	0·395	58·6
	21	9·7	0·489	5·04	0·440	4·46	0·275	61·6
	23	12·0	0·554	4·62	0·430	3·58	0·307	71·5
	24	13·8	0·656	4·76	0·620	4·52	0·391	63·1
	25	9·8	0·384	3·92	0·637	6·78	0·445	69·9
	26	15·3	0·686	4·48	0·921	6·32	0·782	25·0
	29	15·0	0·609	4·06	1·65	11·08	0·879	53·3
		27th and 28th—Butter fat, 40 g. per diem. Weight, 8·75 to 8·78 kilos.						

Utilisation of N.

No. 1 shows results upon an exclusive diet of 200 g. of puppy biscuit, given over a period of five days; the utilisation of the N is 93·8 per cent. of the total ingested. On a diet of cooked meat the total N excreted falls, and as the results in No. 2 show, the protein of meat is readily dealt with by the normal gastric and intestinal secretions, only 3·5 per cent. being lost by the faeces. On a mixed diet of meat, milk and puppy biscuit (No. 3), the total daily excretion of faecal N rises and remains steady at about 1 gram. The absorption figure of 89·71 per cent. is probably lower because of the large amount of starch in puppy biscuit which is available for the immediate supply of energy.

No. 4 shows the effect of a milk diet continued for a period of nine days. During the first two days 700 cc. of milk were ingested, and on the third day of the period the first estimation of N gives a remarkably uniform figure. In the course of the last six days of the experiment 2400 cc. of milk and 448 g. of puppy biscuit were given, equal to 25·44 g. N, of which 3·376 g. were found in the faeces, giving an absorption of 92·70 per cent. For the whole period the utilisation figure for N was 87·2 per cent.

In No. 5, where the diet consisted of 400 cc. of milk with puppy biscuit and lemco, the latter in quantities of from 2–30 g. per diem, the percentage

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N excreted daily is not appreciably altered, but here the utilisation is very good, being 97·27 per cent. This of course may be due to the appetising effect of the added lemco; at any rate it shows that given a good appetite and milk not in excess of the needs of the organism, the protein, fat and carbohydrate of milk will be practically all assimilated.

The protein of milk is well utilised and even the addition to the diet of concentrated protein preparations does not appreciably alter the daily loss of N. The results, as shown in No. 6, where ereptone was added to a diet of meat sufficient in itself, clearly demonstrate the avidity and the ease with which the organism deals with protein. A loss of only 5·28 g. of N on such a nitrogenous diet is remarkable.

A diet of meat and mutton fat given over a period of nineteen days resulted in a slight increase in the dry bulk of the faeces which could be accounted for by the nature of the diet and the longer period between the days of defaecation. As No. 7 indicates, the daily N is slightly increased, but the percentage is within the usual normal variations. The percentage utilisation of N is 96·2.

On a diet of caseinogen and palmine (No. 8) the absorption of N is 95·37 per cent. The addition of cod liver oil to the diet had practically no effect upon N absorption, the percentage N utilisation in No. 9 being 96·5.

The N *of the faeces.*

It is known that the composition of the faeces is not greatly altered by feeding dogs on any of the simple food stuffs. This does not hold good for herbivora. Fritz Voit [1892] by his loop experiments showed that the excretions from the isolated gut were similar in constitution to that obtaining in the whole gut. The N content was almost equal in both as calculated per square metre per 24 hours. The loop however contained a greater amount of fat and fatty acids. From these experiments he maintained that the N of the faeces was largely derived from substances excreted through the wall of the intestine and was therefore a product of protein metabolism.

In my tables it is seen that the N excretion remains fairly constant on any diet which does not consist exclusively of meat, for on such a pure meat diet the N excretion is reduced from the approximate daily loss of 1 g. to 0·5 g. per day, the utilisation varying from 89·71 per cent. to 96·53 per cent.

With milk the N figure averages 1·1 g. per day, but the addition of carbohydrate raises the daily N loss to 1·5 g. per day. Where vegetables

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are added to a large milk diet the N loss is evident, but whether this increase is due to an increased secretory activity or less complete utilisation of N, it is difficult to say. As has been pointed out in Nos. 4 and 5, milk, when given in normal amounts, is best assimilated when such a palatable article of diet as puppy biscuit is added. As with meat so with all concentrated protein foods, the N loss is small, the percentage daily loss varying from 3·50 to 5·28 g., which is equivalent to a total daily loss of from 0·38 to 0·71 g. On a low N diet the utilisation was 88 per cent., while on a high N diet the utilisation averaged 95·89 per cent.

Utilisation of fat.

On a mixed diet the fat absorption varies from 89 to 96 per cent., and depends upon the form in which the fat is ingested. On a diet of puppy biscuit the fat absorption was 94·98 per cent., while on a diet of meat only (No. 2) the absorption was 87·1 per cent. In this latter case the fatty acids recovered from the faeces amounted to 7·6 per cent. of total fat in diet, which is equal to a percentage utilisation of pure fat, estimated as fatty acid, of 92·4. An increase of fat in the diet leads to an increased percentage utilisation, as shown by the results in No. 3, where the absorption figures for fat as total petroleum ether extract and pure fat are 92·98 per cent. and 95·86 per cent. respectively.

In No. 4, where the diet consisted largely of milk, 87·68 g. of fat were given during the last six days of the experiment, of which 5·254 g. were lost, which is equal to a utilisation of 94·0 per cent. For the total period of the experiment the absorption of total fat amounted to 91·82 per cent. and of pure fat to 95·34 per cent., proving as has been done so often that a natural emulsion of fat such as obtains in milk is the best condition for its assimilation. On a mixed diet of milk, puppy biscuit and lemco excellent utilisation figures were obtained, as the results in No. 5 show. The amount of fatty acid recovered from the faeces was 2 per cent. of the total fat given, while the total fat extracted amounted to 3·64 per cent. of the fat ingested.

Mutton fat, which has a melting point of from 49° to 51°, is well utilised even when given in large quantities of 50 to 90 g. a day. This confirms von Noorden's statement that a larger quota of fat in the diet effects a more complete absorption. Here 807·86 g. of fat were given during the period under observation; the meat in the diet accounted for 85·6 g. of fat, making a total of 893·46 g. of fat, of which 23·94 g. were excreted, giving a utilisation of 97·4 per cent. The fatty acid recovered from the faeces amounted to

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1·79 g., which is equivalent to a pure fat utilisation of 98·21 per cent. Munk [1884], feeding dogs with suet alone, obtained results indicating an absorption of 90 to 94 per cent. of fat.

Stearin and palmitin are the chief constituents of several preparations which are made to take the place of butter, such as margarine and palmine, etc. The stearin of these preparations is extracted from "kernel" palm nut oil after the removal of the more liquid portion by hydraulic pressure. The melting point of the palm nut oil is 34°–40°, its chief constituent being palmitin. The small amount of stearin in palmine gives to it that solid consistency which makes it such an ideal preparation for feeding purposes.

During the experiment—No. 8—204·0 g. palmine were ingested and 9·199 g. of fat excreted, which is an absorption of 95·5 per cent.; the pure fat utilisation was 95·6 per cent.

No. 9 shows that the free fatty acid in cod liver oil facilitates the emulsifying and the subsequent absorption of the oil; 360 g. of the oil were given and 3·721 g. of fat and fatty acid were excreted; utilisation, 98·97 per cent. A point of note here is that the percentage fatty acid of total fat extracted is distinctly higher than in the preceding tables, and estimating the utilisation from the point of total fatty acid recovered we find almost a total assimilation, 99·04 per cent.

On a low fat diet the utilisation was 87·1 per cent. and on a high fat diet 97·4–98·9 per cent.

The effect of pancreas feeding.

It could not be expected that the addition of pancreas to the diet in normal dogs would materially improve such absorption figures as 97·27 per cent., etc., which are the result of feeding on a mixed diet composed largely of meat, with the addition of puppy biscuit and a bone, ingredients calculated to bring into play to their fullest extent not only the physical but the psychical factors in digestion.

A dog fed on 250 g. of meat and 150 g. of pancreas for three days excreted at the end of that period 1·84 g. N (5·74 per cent.) and 1·92 g. fat (6·25 per cent.), which is equal to a utilisation of N, 96·15 per cent. and of fat, 97·15 per cent.

Utilisation of Protein N and Fat in Diabetic Animals.

A. *In partially depancreatised dogs.*

Condition of animals. The day after operation Dog I, which had a graft of pancreatic gland in its abdominal wall, weighed 8·6 kilos and excreted

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1·85 g. sugar in the 24 hours sample of urine. On the following day the loss of sugar had risen to 30 g., about which figure it remained for a week, after which for a few days the excretion rose to between 40 and 50 g., subsequently falling to 28 g. and remaining with varying fluctuations at about 30 g. per day till the eighteenth day after operation, upon which day the dog was killed by bleeding under $CHCl_3$ anaesthesia. The D : N ratio in this dog varied from 3·8 : 1 on the third day to 5·4 : 1, which latter figure was reached on the tenth day after operation, the ratio for the following days being between 3·2 : 1 and 5·0 : 1. From these data one sees that despite the graft of pancreatic tissue and the apparently good condition of the animal, a decidedly diabetic condition obtained, in view of which the following figures for utilisation are worthy of note. In comparison with the totally depancreatised dogs the slower loss in weight and the higher respiratory quotient—0·69 to 0·740—show that the disturbance was not so acute as in cases of immediate depancreatisation.

Dog II, in which a portion of the gland was left *in situ*, weighed 8·0 kilos before operation. On the fourth day after operation the sugar excretion had risen to 5 g. in the 24 hours with a D : N ratio of 1 : 0·7, while on the eighth day the total excretion for the 24 hours was 11·0 g., from which time it rapidly fell until only a trace of sugar upon occasional days could be obtained. The loss of weight was very gradual, the respiratory quotient remained normal 0·78 to 1·03 according to diet, and the glycosuria, up to the time the dog was totally depancreatised, was transient.

Utilisation of N. Dog I. In Nos. 10 and 11 (Table II) the N absorption is 93·2 and 95·4 per cent. Pancreas was added to the diet in both cases;

TABLE II.

Partially depancreatised Dogs.

10. Dog I. Partially depancreatised; c graft in abd. wall.

Diet: May 21, meat 100 g.; May 22–June 3, meat, 150 g., pancreas, 50 g.; June 3–8, meat, 200 g., pancreas, 50 g.; June 9 and 10, meat, 200 g., pancreas, 60 g.

Date		Dry wt. of Faeces g.	Total N	% N	Total fat	% fat	Total fatty acid	% fatty acid of total P. E. Extract	Wt. of dog in kilos
May	23	44·0	2·834	6·44	13·53	30·76	10·34	76·6	8·1
	26	14·1	0·852	6·04	4·935	33·76	3·567	70·47	7·60
June	2	35·0	2·264	6·44	10·50	30·10	7·154	72·7	6·80
	6	27·0	1·890	7·0	4·320	16·56	3·604	80·9	6·60
	7	26·0	1·929	7·42	3·640	14·0	2·496	69·0	6·50
	11	24·2	1·829	7·56	2·90	12·16	1·920	66·2	6·20

Weight before op. 9·15 kilos. Op. 18th. Weight 20th, 8·65 kilos.

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TABLE II—*Continued.*

11. Diet: caseinogen, 60 g.; pancreas, 100 g.—19 days.

Date		Dry wt. of Faeces g.	Total N	% N	Total fat	% fat	Total fatty acid	% fatty acid of total P. E. Extract	Wt. of dog in kilos
June	15	17·7	0·977	5·18	1·848	10·44	1·358	73·4	6·0
	16	23·4	1·579	6·72	0·799	3·36	—	—	5·75
	17	24·0	1·310	5·46	2·112	8·88	1·632	77·2	5·70
	19	21·4	1·02	4·76	1·914	8·94	1·519	79·4	5·60
	20	28·2	1·421	5·04	2·989	9·68	1·762	61·9	5·50
	22	36·4	1·783	4·90	4·659	12·20	3·101	69·8	5·45
	23	8·0	0·3584	4·48	1·280	15·76	1·20	94·0	5·25
	26	23·3	0·9786	4·20	6·291	26·82	5·545	87·7	5·10

12. Dog II. Portion of gland left *in situ*; c pancreatic duct intact.

Diet: meat, 100 g.; pancreas, 50 g.; May 21–June 3. Meat, 150 g.; pancreas, 50 g.; June 4–10.

Weight, 8·0 kilos. Op. 18th. Weight, 20th, 7·75 kilos.

May	22	30·5	0·818	9·24	5·155	16·98	4·575	85·1	7·40
	24	12·4	1·160	9·36	1·860	15·5	1·50	80·7	7·20
	26	27·0	2·419	8·96	5·94	22·0	—	—	7·00
	27	40·0	3·080	7·70	4·40	11·4	3·290	74·8	6·90
	28	9·0	0·680	7·56	1·681	18·68	1·07	71·0	6·80
	31	8·5	0·643	7·56	1·275	15·12	1·013	78·8	6·80
June	1	35·0	2·264	6·44	10·5	28·12	7·154	72·7	6·75
	2	5·6	0·450	7·98	0·622	11·1	0·397	65·0	6·75
	3	9·2	0·834	6·44	1·454	15·1	1·097	75·4	6·75
	4	11·5	0·744	6·86	1·242	10·88	0·654	53·0	6·85
	6	16·8	1·15	8·40	1·405	8·36	0·814	60·3	7·00
	8	11·0	0·924	7·70	1·430	12·381	0·782	59·4	6·75
	10	20·5	1·578	8·96	3·895	18·68	2·844	70·9	—
	11	12·6	1·130	7·98	1·385	10·94	0·894	71·0	6·75

13. Diet: meat, 150 g.; pancreas, 30 g. (16th to 20th).

	17	8·5	0·607	7·14	0·822	9·66	0·434	55·5	6·75
	18	13·55	1·021	7·564	1·49	10·90	1·035	66·7	—
	20	11·3	0·886	7·84	1·34	11·04	0·7063	53·0	6·75

14. Diet: meat, 250 g.; pancreas, 50 g.—5 days.

	13	21·5	1·535	7·14	3·775	17·56	1·587	42·0	6·35
	14	18·0	1·285	7·14	2·660	14·80	1·89	74·6	6·40
	16	16·7	1·403	8·40	2·348	14·06	0·902	40·0	6·50

15. Diet: June 30, meat, 100 g., puppy biscuit, 100 g.
Thyroid, July 2–4, 1 g.; 5–6, 1·5 g.; 7–8, 2 g.; 9–10, 3 g.

July	1	—	0·916	—	0·891	—	0·810	61·4	—
	2	23·6	1·784	7·56	2·025	8·58	1·376	68·2	6·60
	3	17·0	1·142	6·72	0·8262	4·86	0·435	52·6	6·60
	4	30·0	2·058	6·86	1·764	5·88	0·936	55·0	—
	5	31·2	2·184	7·0	1·50	4·84	0·775	52·8	6·62
	6	17·0	1·071	6·3	1·221	7·10	0·675	56·8	—
	7	26·0	1·310	5·04	1·70	6·54	1.107	65·1	6·65
	8	39·2	2·566	6·58	2·74	7·0	1·113	41·0	6·60
	9	32·5	2·138	6·58	2·35	7·20	1·170	50·0	6·50
	10	24·7	1·729	7·0	2·021	8·18	1·052	52·1	—
	11	35·8	1·356	6·30	2·899	8·10	1·525	52·6	6·40

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in the former the chief constituent was meat, 150–200 g. per day, in the latter, caseinogen, 60 g. per day. Both these experiments show a remarkable utilisation of N, the protein being presented in a most assimilable form.

Dog II. The figures in No. 12 are the result of a diet similar to, but more generous than, the preceding, and the observation was continued over a much longer period—three weeks. The excretion of N is less; during the period 2490 g. of meat and 890 g. of pancreas were given, which is equal to 102·09 g. and 26·7 g. N respectively, making a total of 128·79 g. N, of which 19·87 g. were excreted, giving a utilisation of 86·0 per cent. The average daily excretion, which was 0·990 g. N, is quite a normal figure.

In No. 13, where the same diet was repeated, there was an improved N utilisation, 91·7 per cent. An increased meat diet with pancreas still maintains an improved absorption, as is shown by No. 14, where the N utilisation was 93·27 per cent.

These high figures can be due to no other cause than the beneficial effect exerted by the added pancreas in the diets, because on a mixed diet of meat and puppy biscuit, which in normal animals leads to improved protein absorption, and regardless of the fact that the protein need of the organism was accentuated by thyroid administered, the digestive system did not on this mixed diet, without pancreas, maintain its previous powers of utilisation, the N absorption falling to 79·86 per cent. (No. 15.)

It is seen that where a portion of the gland is left *in situ* the results are similar to those obtained with a graft of the gland. Most of the previous work has been done irrespective of the addition of pancreas, and in these experiments where no pancreas has been added to the diet, the N loss has varied from 17 to 27·5 per cent.

Utilisation of fat. In Dog I the amount of fat ingested was 209 g. and that excreted 39·82 g., which gives a utilisation of 80·95 and of pure fat 86·1 per cent. The first sample of the experiment contained a large amount of fat, 13·53 g. The second, three days after, which was of much smaller bulk, contained 4·935 g. The percentage of fat in both samples was over 30. These figures are high because of the constipation existing, but with a more regular action of the intestine the total amount of fat per sample approximates more closely to a normal figure, but that fat is being lost is quite evident both from the total and percentage figures. In No. 12 almost the same amount of fat was given, there being double the daily amount of pancreas ingested as in the previous case. Here the absorption figure has improved, amounting to 87·44 per cent. of total fat and 91·7 per cent. of pure

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fat, calculated as fatty acid. While it is possible to get a good absorption of fat, it cannot be maintained for long periods; as can be readily understood, a diabetic organism cannot be expected to show the same adaptability as the normal.

Dog II was fed for three weeks on a meat and pancreas diet. The fat loss was comparatively large, being 23·9 per cent., a figure which is more in agreement with Weintraud's results, but in view of better absorption on other occasions cannot be accepted as being an indication of the failure of the diabetic to absorb fat to any great extent.

On a rather sparing diet of meat and pancreas the amount of fat given was 30 g., of which 3·65 g. were excreted, being equal to a utilisation of 87·83 per cent. estimated as total fat absorbed and of 92·75 per cent. as pure fat. With a more liberal diet similar to the foregoing the utilisation was practically the same, viz. 85·95 per cent. and 93 per cent. (No. 14.)

With partially depancreatised dogs on a diet of meat and pancreas there is an average loss of fat amounting to 14·37 per cent.

On a diet of meat and puppy biscuit without the addition of pancreas, the utilisation of the small amount of fat in the diet is very poor (No. 15), the figures being, for total fat, 72·12 per cent., and for pure fat, 84·7 per cent. A loss of 27·8 per cent. of fat on such a diet should never occur when pancreas is added. In all these cases of partial depancreatisation the excretion of fatty acid with pancreas feeding is 60–80 per cent. of the total petroleum ether extract. The lack of pancreas in the diet results in a lowering of the percentage excretion of the fatty acid to between 40 and 65.

B. *In totally depancreatised dogs.*

Condition of the animals. As several dogs having a graft of pancreas in their abdominal wall or a piece left *in situ* have been kept in this laboratory in good condition for over two months, this preliminary operation, leading to a mild type of diabetes, undoubtedly allows the animal to adapt itself to the new conditions and so lessens the shock of the subsequent total extirpation; it also helps the animal to conserve its energy for a longer time during the much more severe type of diabetes produced by the second operation.

In totally depancreatised dogs there is always some evidence which at first hand leads one to suspect an increased amount of fat in the faeces. On a diet of meat there is an increased faecal odour, due to protein decomposition in the large intestine, and with a diet of fat there is invariably a typically

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fatty foul-smelling stool, which after drying on glass plates presents the appearance of sheets of dark brown gelatin. It is evident therefore that the digestive and absorptive powers will vary according as the dog has been totally depancreatised by two stages or at one operation, because of the sudden onset of severe diabetes and the worse condition of the dog after immediate depancreatisation.

TABLE III.

Totally depancreatised Dogs.

16. Dog III. Total depancreatisation.

Diet: July 19, meat, 100 g., pancreas, 50 g.; July 22, meat, 200 g., pancreas, 100 g.

Date		Dry wt. of Faeces g.	Total N	% N	Total fat	% fat	Total fatty acid	% fatty acid of total P. E. Extract	Weight of dog in kilos
July	21	14·6	1·242	7·70	4·322	29·6	2·073	48·0	5·80
	22	15·6	1·267	8·12	2·833	18·16	2·037	71·9	5·80
	23	9·3	0·890	9·52	1·55	16·66	1·109	71·6	5·70
	24	11·7	0·982	8·40	2·14	18·28	1·183	55·0	5·65
	25–26	28·1	2·469	8·82	4·158	14·80	2·154	57·8	5·60
	27	10·3	0·894	8·68	1·456	14·14	0·731	50·2	5·50
	28	11·1	0·8081	7·28	2·775	25·0	1·607	57·9	5·35
	30	14·6	0·930	6·44	3·35	23·10	1·989	59·0	5·20

Before op. 6·5 kilos. Op. July 17. Weight, July 19, 6·15 kilos.

17. Dog IV. Totally depancreatised.

Diet: Feb. 1, meat, 500 g. for over 3 days.

Feb.	3	34·5	3·236	9·38	8·280	23·90	7·445	89·6	6·8
	4	16·5	1·60	9·94	3·30	20·44	2·673	81·0	5·6

Op. Jan. 30; weight before, 7·0 kilos; Feb. 1, 6·35 kilos.

18. Diet: meat, 160 g.; pancreas, 50 g.; milk, 50 g.—5 days.

	21	18·0	1·323	7·35	3·240	18·46	2·45	75·7	6·75
	22	11·2	0·909	8·12	1·789	17·10	1·590	83·0	6·50
	23	14·6	1·267	8·68	1·752	12·051	1·452	80·0	6·40

After op. 16th, 7·2 kilos.

19. Diet: meat, 600 g.; pancreas, 100 g. (i.e. total).

Mar.	5	12·6	1·12	9·73	2·06	15·37	1·431	70·0	7·6
	6	24·4	1·133	4·76	4·88	19·8	3·618	74·1	7·3
	7	24·8	2·15	8·68	4·037	16·28	2·536	63·5	6·7

Op. Mar. 2. Weight before op. 8·0 kilos; after Mar. 4, 7·5 kilos.

Dog III from which the results in No. 16 (Table III) were obtained was made totally diabetic by the two-stage operation, and a marked glycosuria immediately supervened upon the second operation, the dog eliminating 80 to 100 per cent. of any sugar given to it. The D : N ratio varied between 2·8 and 3·1 : 1, and the total excretion of sugar for 24 hours was between

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30 and 40 g. The loss of weight was at about the rate of one kilo in ten days, which is similar to what obtained in the first partially depancreatised dog. Another sign that a distinct metabolic disturbance had ensued was the low level of the respiratory quotient, the rise of which consequent upon sugar injection was greatly diminished. Dog IV was the subject of severe diabetes, losing weight rapidly and excreting large quantities of sugar. This dog as well as the two following (Nos. 18 and 19) were totally depancreatised at one operation, and, as will be pointed out later, received no pancreas in their diet. The others, though not wasting so rapidly as the former, lost flesh somewhat more quickly than those dogs which were only partially depancreatised. They had a D : N ratio of 3·8 : 1 and upwards, excreted 15 to 50 g. of sugar per diem, and eliminated 85–100 per cent. of sugar added to their diet.

Utilisation of N. The results obtained from a dog which had its pancreas removed in a two-stage operation are given in No. 16. The diet consisted of meat and pancreas and was in excess of the absolute requirements of the organism. The N utilisation here was 92·1 per cent., which is even within the limits of the absorption of normal animals. The following experiment shows very clearly what a detrimental effect immediate depancreatisation has upon the digestive functions of an animal. No pancreas was added to the diet. There was a loss of 21·9 per cent. N given in the food. In contrast to this, No. 18 shows how effective is the addition of pancreas. Over a period of five days 44·0 g. N were given, of which 3·499 g. were recovered in the faeces, which amounted to a utilisation of 92·0 per cent.

It is evident that for good absorption the dog must make a good recovery after the operation. The absorption shown in No. 19 is very poor, due doubtless to the fact that the dog was not in good condition, for this dog showed signs of gastric and intestinal disturbances on the fourth day after operation and died suddenly on the evening of the fifth. The amount of protein ingested as meat was approximately 500 g. and 100 g. of pancreas, equal to 25·05 g. N. 4·413 g. were excreted, giving a utilisation of 82·43 per cent.

Utilisation of fat. The absorption of fat in a dog made diabetic by the two-stage operation was very good, falling very little below the best obtained with a graft of the pancreatic gland. The amount of pure fat recovered from the faeces in this case was 10·06 g., which gives a percentage absorption of 89·94, while for total fat estimated the utilisation figure was 82·7 per cent.

The results of the following experiment (No. 17) are typical of the

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far-reaching disturbance of fat digestion and assimilation consequent upon immediate total depancreatisation, when no pancreas is administered with the food. The total fat recovered was 67·4 per cent. of the amount given, being equal to 32·6 per cent. utilisation.

That pancreas influences fat assimilation to a marked degree is shown by the results in No. 18, where the utilisation figures for total fat and pure fat were 88·3 per cent. and 90·5 per cent. respectively. Once digestive disturbances have set in, all absorption will become deranged, no matter what dietetic means be taken to improve it, e.g. the addition of puppy biscuit or pancreas: this is shown by the poor fat absorption figure of 69·8 per cent. (No. 19.)

Discussion.

These results indicate that the removal of the pancreas causes an immediate and serious disturbance of the digestive functions as regards both protein and fat, and also, as the conditions of these animals show, the power of oxidising glucose is more or less destroyed according to the degree of the diabetes produced.

We see from these results that the figures for utilisation in cases of total depancreatisation with pancreas feeding are somewhat better than in those of partial depancreatisation without pancreas. Still in the total diabetic the function of the absent pancreatic juice must be carried on by other secretions, gastric and succus entericus, as we have no knowledge of any deterrent effect upon the secretions of other juices. Moorhouse [1915] in a recent publication shows that in partially depancreatised dogs there may be an increase of metabolism amounting to 9 per cent., while in total depancreatisation such an increase may vary from 9 to 38 per cent. of the normal. He holds that this increase of metabolism runs parallel to the severity of the case and that it is an expression primarily of interference with carbohydrate utilisation and secondarily with protein and perhaps fat metabolism. The protein destruction is enormously increased, while the actual part taken by protein in the oxidations, i.e. the energy distribution of the proteins, is only slightly increased, 5 to 10 per cent. This increased metabolism would explain the good percentage utilisation of protein and fat in the attempt to make good the great expenditure, when the power of sugar utilisation is more or less lost.

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Conclusions.

1. In normal dogs the N utilisation on a high protein diet varied from 95 to 96·5 per cent., while on a low protein diet it was 88 per cent.

2. Fat utilisation on a high fat diet was 97 to 98·9 per cent., on a low diet 87·1 per cent.

3. In partially depancreatised animals there is very little difference in power of assimilation, whether the piece of gland is grafted in the abdominal wall or left *in situ*, but the addition of pancreas to the diet exerts a most favourable effect in both cases.

Utilisation figures:

With part of gland grafted ..	N = 93–95·4 % Fat = 80–87·4 %	With pancreas
With part of gland *in situ* ..	N = 86–93·27 % Fat = 76–88 %	With pancreas
Without pancreas	N = 79·86 % Fat = 72·12 %	

4. Total depancreatisation leads to an immediate and severe diabetes which is usually terminal in about a week or ten days. This condition is alleviated by performing the operation in two stages, the first being a partial depancreatisation.

5. The addition of pancreas to the food is as beneficial in one-stage operations as it is in the two-stage operation.

6. Immediate depancreatisation without pancreas in the diet produces grave disturbances of digestion and absorption, resulting in a loss of 22 per cent. of N and 67·4 per cent. of fat. The addition of pancreas is most beneficial, the utilisation of N rising to 92 per cent. and of fat to 70–88 per cent.

7. The deleterious effects of extirpation of the pancreas on fat absorption and nitrogen metabolism are thus by no means so serious as has been found by other observers quoted in the introduction to this paper.

I am greatly indebted to Professor Starling for performing the various operations upon these dogs, and for his advice and interest in the work.

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XV. A NEW TEST FOR REDUCING SUGARS IN URINE.

By WILLIAM CRAMER.

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The test depends upon the reduction of mercuric oxide in a weakly alkaline solution to metallic mercury. The degree of alkalinity is an important factor, as the test becomes more sensitive but less specific the greater the alkalinity of the reagent. The reagent is prepared as follows: 0·4 g. mercuric oxide (red or yellow) and 6 g. potassium iodide are dissolved in 100 cc. water. This solution is weakly alkaline. The alkalinity must now be so adjusted that 10 cc. of the reagent are neutralised by 2·5 cc. of n/10 acid, using phenolphthalein as an indicator. This is done by titrating 10 cc. of the reagent with n/10 acid and, after the alkalinity of the reagent has thus been determined, adding the requisite amount of n/10 acid or alkali to the bulk of the reagent. The reagent is a clear, colourless solution which turns slightly yellow on heating and becomes colourless again on cooling. It must remain quite clear on boiling. The reagent thus prepared will be referred to in this paper as the "2·5 standard reagent" in order to indicate its alkalinity.

Potassium cyanide may be used as a solvent for mercuric oxide, but potassium iodide has been found to be preferable, and all the data given in this paper refer to the reagent prepared with potassium iodide.

With this reagent the test if applied to an aqueous solution of a sugar is carried out as follows: 3 cc. of the reagent are heated in a test-tube to boiling. The solution remains clear, but turns slightly yellow. 0·3 cc. of the sugar solution is now added and the mixture again heated to boiling. The test-tube is then removed from the flame. The mixture darkens and a black precipitate of finely divided mercury appears. At first this precipitate gives the appearance of a turbidity, especially if the amount of sugar present is small. On standing, the precipitate soon settles at the bottom of the test-tube.

The test is positive with sugars which give the other reduction tests such as lactose, maltose, xylose and arabinose. It is negative with cane sugar.

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An indication of the sensitiveness of the test is given by the fact that a slight reduction can still be obtained with one milligram of glucose if an aqueous solution of glucose is used.

If the test is applied to normal urine in the way to be described below, a slight but distinct reduction is obtained comparable in degree to that obtained with aqueous solutions of glucose containing 0·1 per cent. to 0·2 per cent. of glucose.

The alkalinity of the reagent has been fixed at the standard given above for the following reasons. The greater the alkalinity of the reagent the greater is the degree of a reduction produced by a given amount of a reducing sugar, so that the reagent could be made more sensitive by increasing its alkalinity. If, however, the reagent is made strongly alkaline, it ceases to be specific for reducing sugars and chemically allied substances, and is reduced by other organic substances, such as creatinine. And since sugars have to be identified as a rule in fluids such as urine and serum containing other organic substances, the reduction of the reagent by substances other than reducing sugars and chemically allied substances must be excluded, if the test is to be an improvement on Fehling's test, where the reduction by substances such as creatinine and uric acid constitutes a serious fallacy, particularly if small amounts of sugar are being tested for.

The alkalinity of the reagent was fixed at the 2·5 standard because at that point the reduction produced by normal urine is very slight and corresponds in degree to that produced by aqueous solutions containing 0·1 per cent. to 0·2 per cent. of glucose. It is then certain that the reduction produced by normal urine is due only to the glucose which it contains and not to any other constituent. This was confirmed by testing the reagent with a number of organic substances, present normally, or likely to be present, in urine, such as urea, uric acid, urates, guanine, allantoin, creatinine, hippuric acid, taurine, bile salts. In amounts exceeding those possibly present in urine all these substances gave negative results.

When the alkalinity of the reagent is increased so that 10 cc. of the reagent are neutralised by 3 cc. n/10 acid ("3·0 standard reagent"), normal urine gives a reduction slightly greater than that produced by 0·1 per cent. to 0·2 per cent. solutions of glucose. It does not necessarily follow that this greater reduction is due to some normal urinary constituent other than glucose. For the 3·0 standard reagent shows no reduction when tested with the urinary constituents mentioned above in concentrations exceeding those found in normal urine. The observations recorded in a previous paper [Cramer, 1915] suggest the possibility that, just as in the case of cupric

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oxide, urine presents conditions more favourable for the reduction of mercuric oxide by glucose than a pure aqueous solution. But in order to exclude possible fallacies the 2·5 standard reagent is recommended and not the slightly more sensitive 3·0 standard reagent.

If, on the other hand, the alkalinity is reduced so that 10 cc. of the reagent are neutralised by 2 cc. n/10 acid ("2·0 standard reagent"), normal urine gives practically negative results, and a distinctly positive result is obtained only if the urine contains as much as about 0·3 per cent. of glucose. An example of the differences obtained with the reagents of different degrees of alkalinity is given in Table I.

TABLE I.

Glucose-solution	"2·0 Standard"	"2·5 Standard"	"3·0 Standard"
0·1 %	hardly visible	very slight	slight
0·2 %	slight	slight	slight
0·5 %	distinct	marked	intense
1·0 %	intense	intense	intense
Urine containing glucose			
0·1 % (normal urine)	hardly visible	very slight	distinct
0·2 %	hardly visible	slight	distinct
0·3 %	distinct	distinct	intense
0·5 %	distinct	intense	intense

There is, of course, no difficulty in recognising the presence of large amounts of reducing sugars by this reagent whether the "2·0 standard," the "2·5 standard" or the "3·0 standard" is used. But the value of this reagent lies in the fact that it enables one to recognise readily very small quantities of sugar in urine, when Fehling's test gives only doubtful results, while Nylander's test is less sensitive and requires for the identification of small amounts prolonged boiling which is often troublesome. A comparison of

TABLE II.

Specimen of urine	Glucose %	Fehling	Nylander	"2·0 Standard"	"2·5 Standard"	"3·0 Standard"
M	0·07	negative (transparent green)	negative	very slight	distinct	distinct
C 1	0·2	doubtful (opaque greenish brown)	doubtful	hardly visible	distinct	distinct
C 2	not determined	probably positive (transparent brown)	distinctly positive	distinct	distinct	distinct

the different tests is given in Table II. The tests were made with three different specimens of urine from two normal individuals. The amount of glucose present was determined by Bertrand's method, using the modification devised by MacLean and Gardiner [1914] for small quantities of glucose.

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As Table I shows, the test here described enables one to recognise not only the presence of small amounts of reducing sugars, but also slight differences in the amounts of sugar present, when these amounts are small. But in order to do so it is necessary to carry out the test in such a way that the conditions under which reduction is produced are constant and that the turbidity, which is a measure of the amount of reduction, is due entirely to the precipitated mercury and not to the phosphates, which are, of course, also precipitated when the urine is added to the hot alkaline reagent. The turbidity due to phosphates can be excluded simply by acidifying the mixture, after the test has been carried out, with acetic acid, which dissolves the phosphates. Since the addition of acetic acid stops the reduction which would otherwise proceed in the hot mixture, it is necessary to add the acid after a fixed interval, say 30 seconds. As further the precipitate of mercury is heavy and rapidly falls to the bottom of the test-tube, the degree of turbidity must be judged at once. In the table the degree of turbidity is described as "intense" when ordinary print cannot be read through the mixture. The terms "slight" and "distinct" are applied when print can be read with ease through the mixture. The term "marked" means that print can be read only with difficulty. This "print reading test" is a useful criterion of the degree of reduction produced by small quantities of sugar. As stated above it must be applied immediately after the addition of the acetic acid.

The test, then, as applied to urine, which must, of course, not have undergone ammoniacal fermentation, is simply as follows: 3 cc. of the 2·5 standard reagent are heated to boiling in a test-tube (of ordinary dimensions). The reagent must remain clear. 0·3 cc. of urine is added and the mixture again heated to boiling. The test-tube is removed from the flame and, after thirty seconds, the mixture is acidified with a few drops of acetic acid. The test-tube is at once held over ordinary print. If the urine is normal, a slight but distinct turbidity remains and print is clearly readable through the mixture.

It may be suggested that the "2·0 standard reagent" is more suitable for clinical use as it gives a negative result with normal urine. But there is no reason why the small amount of glucose present normally in urine should be deliberately ignored, if it can be readily recognised. Moreover, the differences in the degree of reduction given by urines containing a slight excess of glucose (0·2 per cent. to 0·5 per cent.) are better marked with the "2·5 standard reagent" than with the less sensitive "2·0 standard reagent."

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The reduction of mercuric salts by sugar in alkaline solution has been used before by Knapp [1870] as a method for the quantitative estimation of sugar. His solution consists of a strongly alkaline solution of mercuric cyanide. The method has not, however, come into general use, at least not for biochemical work. For the solution is stated [Browne, 1912] to be reduced not only by sugars but also by creatinine, glycerol and a number of other organic substances other than sugars, and is therefore unsuitable for biochemical work either as a qualitative test or as a quantitative method. The same objection applies to the modifications of Knapp's solution by Sachsse and Bauer [quoted by Browne, 1912]. From the observations recorded in this paper it would appear that the non-specific nature of Knapp's solution is due mainly to its strong alkalinity. Indeed, if the reagent described in this paper is made very strongly alkaline by adding strong caustic soda, it is readily reduced even by glycerol. Observations are at present being carried out to determine whether the reaction described in this paper is suitable for the quantitative estimation of sugar in urine and other physiological fluids.

In conclusion the advantages of the test described in this paper in comparison to Fehling's test and Nylander's test as applied to urine may be stated. They are as follows:

1. The test is free from the fallacies inherent in Fehling's test as the result of the reducing action of uric acid and creatinine on Fehling's solution.

2. It is more easily carried out than Nylander's test, especially when small amounts of sugar are tested for.

3. With small quantities of sugar it is easier to judge roughly the amount of sugar present from the degree of turbidity obtained in this test than from the changes observed in Fehling's or Nylander's test. The test is in fact particularly suitable when the amounts of sugar present exceed the normal amount only slightly; that is to say in those conditions where Fehling's test gives ambiguous or doubtful results.

4. It is more sensitive than Fehling's or Nylander's test and allows a distinction to be made between the amount of sugar normally present in urine and that slightly exceeding the normal.

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XVI. ON A GREATLY IMPROVED HAEMIN TEST FOR BLOOD, WITH NOTES ON SOME RECENTLY PROPOSED METHODS.

By WILLIAM BEAM and GILBERT ARTHUR FREAK.

(*Received February 24th, 1915.*)

Since Teichmann in 1853 brought forward his test for blood by the production of haemin crystals, a host of modifications have been suggested by as many writers, and a fresh crop of the same appears each year. This in itself is sufficient indication that the method in its original and modified forms is far from satisfactory. Indeed, even an experienced and skilful worker may, apparently most unaccountably, fail to obtain a positive result, though working with fresh and unaltered blood specimens. With those encountered in ordinary laboratory practice, often minute in quantity and altered by age, the action of heat or sunlight, or the presence of rust or earth, or consisting of stains partly washed out by soap and water, the difficulties and uncertainty are increased to such an extent that in many laboratories the method has fallen more or less into disrepute. A number of sorting tests, presumptive only, have taken its place, the final decision resting upon the detection of corpuscles by the microscope, some form of spectroscopic examination—often exceedingly difficult with the limited amount of material at hand—or, in certain cases, the use of "precipitin" reagents.

None of the above-mentioned sorting tests, e.g. those employing guaiacum, benzidine, leuco-base of malachite green, phenolphthalin, etc., can satisfactorily replace the haemin test, since the latter alone affords absolute and unmistakable proof of the presence of blood.

In a hot dry climate like that of the Sudan, blood stains alter more rapidly than in more temperate zones, and the difficulties experienced with the haemin test are therefore usually greater. The need for some more reliable method than any of those yet advanced was actually felt, and in

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the investigation which was consequently undertaken, the following considerations were kept in mind.

(*a*) The amount of blood present being usually small, it is essential that the whole of the portion taken be brought into solution. To insure this the acid must remain in contact with the suspected material for a long time—some hours at least.

(*b*) For the reason already stated above, the operation must be carried out so that no loss may occur.

(*c*) In many cases, especially when the blood is mixed with rust, earth, etc., it is highly desirable that some means be provided for filtering the liquid.

(*d*) If crystals of good size are to be obtained from so small a bulk of liquid—a few drops at most—special precautions should be taken to insure slow evaporation. Slow evaporation has often been recommended but the means prescribed for securing it have been totally inadequate, the operation being completed in, at most, a few minutes. Our endeavour has been to extend this to hours or even, if necessary, to days.

All the above essentials have been happily provided for in the following simple procedure the success of which, in all classes of cases, has exceeded our most sanguine anticipations.

A small quantity of the suspected material is placed at the bottom of a flat arsenic sublimation tube about 6 by 3 mm. and 35 mm. long. A thin round ignition tube will answer if no flat tube is procurable, but for examination under the microscope the flat form is obviously better. A few drops of acetic acid containing from 0·01 to 0·1 per cent. sodium chloride are added and a very fine cotton thread adjusted so that its upper end is near the top of the tube and the lower end reaches to the bottom of the liquid. The thread should be everywhere in contact with the tube, to which it adheres readily by being moistened with the liquid (Fig. 1). The adjustment is readily made by means of a glass rod one end of which is drawn out for the purpose. The tube is now placed in a rack, or supported by forcing its base into a small blob of plasticine, and allowed to remain until crystallisation has taken place.

Before placing the thread into position, the solution of the blood may be aided, if desired, by cautiously heating the tube over a small flame, loss of the liquid by explosive boiling being prevented by holding the finger tightly over the mouth of the tube.

While such heating appears rarely to be essential, the long contact with

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the acid in the cold sufficing for solution in practically all cases, it is nevertheless advisable in that it gives at once an indication of the strength of blood solution obtained. For the best results this should be quite weak.

The clear liquid, filtered by its passage through and along the thread, is slowly drawn up, by capillary action, to the mouth of the tube. Under these conditions evaporation takes place so slowly that, even when the temperature of the atmosphere is above 38° C., it is complete (if the tube is held in an upright position) only after 12 to 24 hours or more. The rate

Fig. 1. Actual size.

Fig. 2. × 25.

of evaporation is readily controlled, within wide limits, by altering the dimensions of the tube and the angle at which it is supported.

The crystals usually begin to appear, on or near the upper half of the thread, and of sufficient size to be distinguished with a power of 75 diameters, in about an hour; and they ultimately become so large that, in place of requiring a magnification of 250 to 300 diameters, as usually recommended, they may readily be seen with one of 25 diameters or even, in many cases, with a good hand lens (Fig. 2). The crystals are best viewed against a dull white background. A piece of white paper is suitable.

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The extraordinarily large size of the crystals is due, in great part, to the slow evaporation of the liquid; but it is also a result of the removal, by the thread, of the interfering action of the blood albumin on crystallisation. Examination of the thread at the end of the experiment shows that the soluble, non-crystallisable, constituents of the blood are carried to its upper end, and that the maximum growth of haemin crystals is found lower down.

If the solution is sufficiently weak the first crystals form at the point of greatest concentration—the upper end of the thread; but as the albuminous matter accumulates these cease to grow and the largest and most numerous crystals are found, later, about midway down the tube.

For the best results the following precautions should be observed.

The tube should be thoroughly clean and dry. It is best cleaned by swabbing out successively with acetic acid, water, and alcoholic soda, followed by thorough rinsing with distilled water and drying on a hot plate.

The thread should be clean and as fine as can be obtained. A good plan is to take No. 80, "six cord sewing machine cotton" and to untwist it, using one of the "cords" for the test.

The solution of the blood in acetic acid should not be concentrated, but very weak. The liquid should only be faintly pink. This insures large crystals and clean fields.

If the method is to be applied experimentally to fresh blood, the latter must be completely dried before the addition of the acid, and especial care should be taken to work with very dilute solutions.

The test has been applied as described to:

(1) Human blood stains—fresh.

(2) Human blood mixed with earth—a forensic case.

(3) Human blood stains—10 years old.

(4) Sheep's blood stains—fresh.

(5) Sheep's blood stains—18 months old.

(6) Sheep's blood stains, heated to 110° C. for 1 hour.

(7) Sheep's blood stains on a rusty knife, two months old and also exposed to the air and sun for several days.

(8) Sheep's blood stains which had been, in great part, removed by washing with soap and water.

(9) Cow's blood stains—12 years old.

(10) Gamoose's blood stains—12 years old.

(11) Dog's blood stains—two months old.

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Upwards of 300 trials have been made, using the method as described or slight modifications of it, and in no single case did the crystals fail to appear.

Many trials were also made with modifications employing other acids in place of acetic and other forms of halide. These experiments need not be recorded in detail, but the following are some of the conclusions arrived at.

Acetic acid without any saline addition will suffice for stains, fresh or old, which have not been treated with water, the salts present in the blood being sufficient for the test. Stains thoroughly washed with water will not respond unless some salt, chloride or bromide, is added.

As halides, the following were tried. To make the comparisons conclusive the blood stains were previously thoroughly extracted with water. Unwashed stains were also employed.

(*a*) Hydrochloric acid and hydrobromic acid.

(*b*) Sodium fluoride.

(*c*) Ammonium, sodium, potassium, calcium and magnesium chlorides.

(*d*) Ammonium, sodium and potassium bromides.

(*e*) Sodium and potassium iodides.

Hydrochloric and hydrobromic acids gave invariably negative results.

Sodium fluoride gave positive results in some cases, but inferior to those with chlorides or bromides.

All the chlorides and bromides tried gave good results. With magnesium chloride and calcium chloride (one tenth per cent. solutions) there is a marked tendency to the formation of a greater number of crystals, of smaller size but long and very slender.

The iodides, with washed blood stains, usually gave negative results. In some cases a very few small crystals were formed but these appeared to be due to the incomplete removal of chloride from the blood, or its presence as impurity in the reagent. With unwashed stains iodides gave results inferior to those with chlorides or bromides. Similarly a reagent composed of a mixture of the three halides gave results in some cases negative and, in all, inferior to those with chloride or bromide alone. From these results the conclusion appears obvious that the presence of iodide in the reagent is distinctly contra-indicated.

As substitutes for acetic acid, formic and propionic acids were tried. Lactic acid, mentioned by Teichmann and recommended recently by Symons (*vide infra*), is obviously not suitable for this form of the test as it is not sufficiently volatile.

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The results with formic acid, alone and with the addition of the various halides mentioned above, were invariably negative.

Propionic acid gave results, in some cases negative, and in all cases inferior to those with acetic acid.

In short, of the solvents experimented with, acetic acid was found to be by far the best and is in fact, for this form of the Teichmann test, the only reliable one. Further, no appreciable advantage was found to result from the substitution of other forms of halide for sodium chloride.

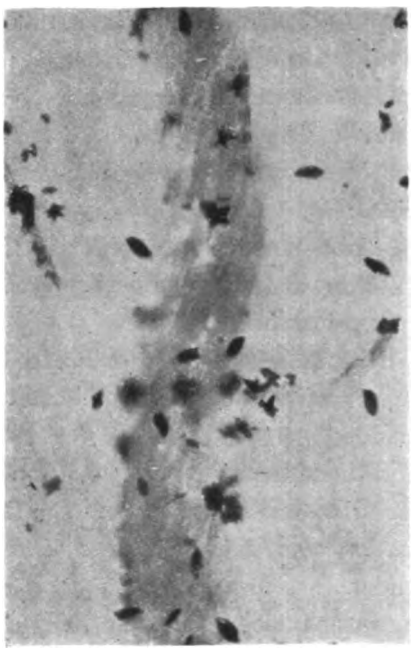

Fig. 3. ×50.

It was noted that in the case of very old blood specimens there is a marked tendency to the formation of short, broad haemin crystals and also of hemp seed forms (Fig. 3).

Notes on some recently Proposed Methods.

Nippe [1912] recommends the following reagent as affording a "ready and certain" means for the identification of blood stains: potassium bromide 0·1 g.; potassium iodide 0·1 g.; potassium chloride 0·1 g.; glacial acetic acid 100 g. A drop of the solution of the stain in the reagent, on a

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microscope slide, is covered with a cover glass and warmed until bubbles just form. "As it cools the growth of the haemin crystals can be watched under the microscope."

It is not easy to see any scientific basis for this modification of the reagent. In our hands it has been found to offer no advantages whatever and the method as a whole suffers from all the disabilities of the original procedure. In the light of the experiments already detailed it would seem likely that any positive indications were obtained, not as a result of the presence of iodide, but in spite of it.

W. F. Whitney [1912] published a note on a method of performing the haemin test which appeared to have advantages. It was noted that formic acid of sp. gr. 1·2 when placed on a glass slide and allowed to evaporate, disappeared gradually from the periphery to the centre. A drop of acetic acid, on the other hand, did not hold together but spread over the slide and evaporated instantly. Formic acid appeared therefore to be a better solvent for the blood, as it permitted of concentration of the solution and the deposition of the crystals in a much more confined area. The method recommended was as follows. A minute fragment of the suspected substance —a scraping from a stain or a bit of stained fibre—is placed on a slide and a small drop of formic acid applied with a glass rod. Over the drop is inverted a small watch glass which should not touch it nor project beyond the edge of the slide. Gentle heat is applied beneath the drop and the moment condensation is seen inside the watch glass, the heat is discontinued, the watch glass removed, and the remainder of the liquid allowed to evaporate in the air. When dry the spot is examined under the microscope. The crystals are said to be small, requiring a high power objective to see them well, but so numerous that they cannot be overlooked.

The remarkable statement is added that "the fact they are so small shows that only a minute quantity of blood is necessary for their formation."

We have made many trials of the above method with most disappointing results. Haemin crystals were obtained in one case using quite fresh blood of which a larger quantity was taken than is usually available for test. Aside from this all trials were fruitless, although the field was searched most carefully under a 1/7 inch objective. Even when precautions were taken to insure much slower evaporation than is possible with the method as detailed, the issue was still wholly unsatisfactory.

These results bear out those obtained by the method detailed in the body of the paper which indicate that formic acid cannot safely be substituted

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for acetic acid for the haemin test. This point was very thoroughly investigated by the use not only of formic acid alone but of formic acid containing hydrobromic acid, sodium fluoride, sodium chloride, potassium bromide and potassium iodide. In no case were haemin crystals formed either with fresh or with old blood stains.

An improvement on the procedure as recommended by Whitney was effected by substituting for formic acid a mixture of equal parts of formic acid and acetic acid, the test being applied as described except that the slide is best slightly heated before the drop of liquid is placed upon it. The results were, however, at best, unsatisfactory and the method failed entirely in the case of old stains or those which had been partially washed out.

A far better method than either of the above, but taking advantage of the same principle of prevention of spreading of the liquid, is the following. The solution of the suspected material is effected in a very small test tube, such as an ignition tube, the reagent being a few drops of acetic acid containing 0·01 per cent. of sodium chloride. The tube should be heated cautiously over a small flame, or for ten or fifteen minutes in a steam bath or oven, in order to insure complete solution. The liquid is then transferred by means of a small pipette to a glass slide coated with a very thin layer of paraffin wax, and set aside in a place protected from dust, for evaporation. The amount of wax on the slide should be so small as to be only just visible. The slide may be prepared by melting on it a small quantity of wax, the excess of which is then removed by means of a dry cloth.

The rate of evaporation of the liquid will depend upon the amount of paraffin wax on the slide. It has been found that the wax dissolves in the acetic acid, and after the first rapid concentration has taken place the liquid becomes coated by a continuous film of wax which checks evaporation to such an extent that it is in some cases not complete even after two or three days. Under these conditions the crystals formed are usually few in number but very large. With less paraffin wax present, so that evaporation is complete in about an hour, crystals are formed of sufficient size to enable them to be distinguished by a magnifying power of about 50 diameters.

The method has been found to be applicable even to very old blood stains, which points clearly to the fact that the difficulties experienced with the ordinary haemin test, when so applied, were not due, as has been frequently claimed, to decreased solubility of the blood, but rather to too rapid evaporation of the solvent.

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The method is, as stated, a marked improvement on the ordinary one; but it is not intended to recommend it in preference to that described above, which is far superior to it on the score both of reliability and delicacy.

C. T. Symons [1913] recommends the following modification of Teichmann's test as especially suited to old stains which have been exposed for a long time to air and sun in the tropics: "Solid sodium iodide, freshly prepared, is dissolved in lactic acid (sp. gr. 1·2) to about one per cent. The solution becomes brown on keeping but this change does not appear to impair its action, at any rate for some months. The fragment of stain is covered with the solution, the cover glass is placed in position and the whole slowly warmed to such a temperature that the solution is just about to boil under the cover glass. In most cases five minutes' treatment is sufficient to produce crystals. If they have not then appeared the heating must be continued; more solution must be allowed to run under the cover glass if necessary, but this is not often the case as the lactic acid does not evaporate as rapidly as acetic acid. The resulting crystals differ very much in size in different cases, being usually rather small, but dark and perfectly characteristic."

In our hands this method gave, at first, very unsatisfactory results. It failed entirely in the case of old stains and even with quite fresh specimens positive indications were by no means the rule. Later, using another sample of lactic acid, much better results were obtained. It was found however that success was to a great extent dependent upon the control of the heating. A small water oven containing a saturated salt solution (boiling at about 107° C.) is probably the safest means, but the temperature of an ordinary water oven is usually sufficient. The best results were obtained not, as recommended, by long heating but by heating for about five minutes only. In some cases, when heating was prolonged, what appeared to be crystals too small for positive identification were formed, but disappeared entirely on further heating. Success in ordinary cases appears to depend upon the entanglement of a supersaturated liquid in the meshes of the fabric. In other words if the blood material is allowed to become diffused throughout the entire mass of the liquid under the cover glass, no crystals may be formed.

"Freshly prepared" sodium iodide is prescribed for the reagent. In our experience this has not been found to be necessary, the salt which had been kept a long time answering equally well.

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Summary.

(*a*) Of the reagents which have been recommended for use in Teichmann's test for blood, acetic acid is by far the best and is the only one on which entire reliance may be placed if the method by evaporation is employed.

(*b*) Acetic acid, without salt of any kind, suffices for the test with blood stains, fresh or old, provided the stains have not been extracted with water. As a precautionary measure it is best however to use a reagent containing a minute proportion—about 0·01 per cent.—of sodium chloride.

(*c*) No advantage results from the substitution of other salts for sodium chloride in the acetic acid reagent.

(*d*) Iodides may not be used to substitute chlorides or bromides in the above. This does not apply to the reagent made with lactic acid.

(*e*) Where positive results have been obtained from the use of an acetic acid reagent containing iodide, the effect is apparently due to the chloride naturally present in the blood, or as impurity in the reagents.

(*f*) The difficulties experienced with Teichmann's test when applied to stains, both fresh and old, are due chiefly to the too rapid evaporation of the solvent and, to a less extent, to interference of the albuminous matter of the blood with the crystallisation. Evaporation should be extremely slow and when carried out in the manner detailed, which also eliminates the harmful effect of albumin, crystals are obtained with the greatest certainty and of remarkably large size, even though only a minute amount of blood be present.

(*g*) The test as described was found to be equally applicable to blood stains fresh or old (12 years), stains partially removed by soap and water, or heated to 110° C., or mixed with earth, or to old stains on rusty iron which had been exposed to strong sunlight and atmospheric conditions for several days.

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XVII. A STUDY OF THE METABOLISM IN EXPERIMENTAL DIABETES.

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I. Introduction.

The diabetes produced in dogs by the removal of the pancreas has been the theme of many investigations which have shown how similar the condition is to a severe type of human diabetes and have given hope of an explanation upon similar grounds. The questions which investigation has left debatable concern the relative importance of two factors which might be primary causes. These factors are:

1. An over-production of sugar by the organism.
2. A loss of the power to use sugar by the tissues.

According to the over-production theory the removal of the pancreas does away with a normal control exerted upon protein and fat metabolism and results in a flooding of the organism with sugar formed from these substances so that the limit of sugar usage is exceeded and a portion of the sugar wasted. The non-utilisation theory presupposes that the internal secretion of the pancreas is indispensable to the tissues in the combustion of carbohydrate. The over-production theory might well be accepted as a working hypothesis but for certain objections. These are the low level of the respiratory quotient, which points to non-utilisation of carbohydrate although the blood is hyperglycaemic, and the steadily increasing D : N ratio in the course of the diabetes when no carbohydrate is given. It is also very plain that the over-production theory would be difficult to prove until the question of the utilisation power had been settled.

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The problem of utilisation has been attacked in three ways. The first method was to administer sugar and see how much of the dose could be recovered from the urine. Minkowski based his conclusion as to the non-utilisation of glucose upon results from this method, and his observations have been confirmed many times. The second method consists in observations upon the blood-sugar content. Starling and co-workers estimated the amount of sugar used by the heart by the fall in blood sugar level during an experiment with the heart-lung preparation. In the first series of experiments of this type Knowlton and Starling [1912] found evidence of a loss of the power of sugar utilisation by diabetic hearts. Later work has shown that sugar disappears from the blood perfusing diabetic hearts and has pointed out the possible sources of error in experiments of this type [Patterson and Starling, 1913; Macleod and Pearce, 1913]. Whether conclusions as to sugar usage can be safely made from the disappearance of reducing substance from the blood in such experiments is still a matter for consideration. The third method of investigating sugar utilisation in diabetes is by observing the respiratory exchanges of the intact animal or isolated tissue. The determination of the respiratory quotient gives a definite measure of the extent to which carbohydrate is being made use of by the organism. Evans and Starling [1914] have studied the respiratory exchanges of the heart-lung preparation in a series of normal and diabetic dogs. They find that the respiratory quotient of the diabetic preparation is lower than the normal, taking averages of the large series of observations. They conclude that the diabetic heart shows little evidence of sugar utilisation because of the low level of the respiratory quotient in spite of the fact that the blood is hyperglycaemic. Porges and Salomon [1910] maintain that the carbohydrate utilisation is unimpaired, since they observed high respiratory quotients in diabetic dogs whose abdominal organs were excluded from the circulation. Murlin, Edelmann and Kramer [1913] point out, however, that a high respiratory quotient in such a condition is of no significance as regards sugar usage. It is evident, as these authors state, that the abrupt change in circulatory conditions, coupled with respiratory and blood reaction variations incident upon the clamping of the abdominal vessels, must be followed by an abnormal respiratory exchange. In the extensive observations of Benedict and Joslin [1910, 1912] upon human diabetes the severity of the disease has been found to increase with the lowering of the respiratory quotient. The respiratory exchange in diabetes indicates a loss or an impairment of the power of sugar utilisation, at least in the light of our present knowledge.

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In view of the unsettled state in which the question of sugar usage in diabetes still remains it seemed desirable to undertake an investigation of the metabolism in dogs before and after depancreatisation. Observations were made upon the respiratory exchange of these animals and detailed analyses of the urine and faeces carried out. The main objective has been the question of sugar utilisation. The results are reported in the following paper.

In this report we wish to acknowledge gratefully the considerable assistance we have received from the members of the staff and other workers at the Institute of Physiology. Dr Plimmer and Mr Reeve have done the urine analyses throughout the research. Dr Cruickshank carried out the tissue and faeces analyses. We take pleasure in thanking these and all the workers at the Institute for help in every stage of the research. We are greatly indebted to Professor Starling, at whose suggestion the work was undertaken, for performing the necessary operations and for his help and advice throughout the research[1].

II. Methods.

Animals. Bitches were used in all the experiments. Care was taken in the selection of young and normal animals and in training them to lie quiet in the apparatus. While under observation the dogs were kept in metabolism cages in a well-lighted, heated and ventilated room.

Feeding. The animals were usually fed once a day at about 5 p.m., no food being left in the metabolism cage over night. The food given was carefully weighed and precautions were taken to prevent wasting of the weighed amount.

Diets and Food Analyses. The diet given to normal dogs varied in character for purposes of experiment. Full notes of the diet will be found in the protocols. The standard diet given to diabetic animals was chopped horse meat and ox pancreas, in the proportion of four to one. Samples of the food were analysed from time to time and estimations of total nitrogen, carbohydrate, phosphate and fat were made where necessary. The following table gives these analyses and the corresponding abbreviations will be used in the protocols when referring to these foods.

[1] Dr Plimmer wishes to thank the Government Grant Committee of the Royal Society for the grant defraying the expenses of the chemical work in connection with this research.

The expenses in connection with the analyses of fats were partially covered by a grant from the Royal Society to Dr Cruickshank.

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TABLE I.

	Abbreviation	N %	Carbohydrate %	P_2O_5 %
Horse meat	M.	4·0 to 4·4	—	0·5 to 0·66
Ox pancreas	P.	2·89	—	1·16 to 1·30
Caseinogen	C.	11·0	—	—
Gelatin	G.	14·0	—	—
Puppy biscuit	P.b.	3·2	60·0	—
Palmine	Pal.	—	—	—
Cod liver oil	C.L.O.	—	—	—

Sugar administration. The sugars administered were glucose and fructose. The dose given was 20 g. by the mouth, as the dogs were found to take this amount dissolved in water readily, and during succeeding respiratory observations would remain quiet in the apparatus. Rectal enemata of sugar solutions were also given, but owing to difficulties in controlling the amount given, liability of the enemata being expelled and varying rate of absorption, the results cannot be regarded as regular enough for comparison. With intravenous injections difficulties arose from the subsequent uneasiness of the animals in the apparatus. The effects of intravenous sugar on the respiratory exchange of diabetic animals will be studied in a future research. The standard dose of glucose or fructose in these experiments was 20 g. given per os.

Operations. The operations were all performed by Professor Starling. The dogs were given a small dose of morphine about two hours before the operation and full anaesthesia was produced by ether-chloroform mixture. Due aseptic precautions were taken and no serious wound infections were observed. All the animals recovered quickly from the operation and showed no uneasiness on the following day.

The method of Hédon was employed in the total extirpations of the pancreas and six dogs were operated upon in this manner. They were dogs I, II, III, IV, V, VI.

Two dogs, VII and VIII, were partially depancreatised and as the procedure was different the operations will be described separately.

Dog VII. All pancreatic tissue was removed from the abdominal cavity, leaving a small portion of the tail, about one square centimetre, free with its blood supply intact in a pedicle of mesentery. This portion was implanted under the skin of the abdominal wall. The graft was functional, because there was a discharge of clear juice for a short period after the operation. This juice digested fibrin slowly. The appearance of sections of the graft is

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noted later. In histological structure the tissue showed little evidence of degeneration a month after the operation.

Dog VIII. This animal had two operations performed on it.

Operation 1. All the pancreatic tissue was removed with the exception of a mass about the size of a pea, which was left close to the main pancreatic duct. The duct was ligated on the peripheral side of the mass. This small portion was evidently functional as the dog, after having a slight glycosuria for a week, became apparently normal and only showed a slightly reduced sugar tolerance for glucose and fructose.

Operation 2. The remainder of the pancreas was removed and the abdominal wound again closed. The dog immediately developed a marked glycosuria with a D : N ratio of 3·2, but at the end of two weeks seemed in far better condition than is usually the case with totally depancreatised animals.

Collection of urine and faeces. The dogs were kept in metabolism cages while under observation. They were catheterised at the end of twenty-four hour periods at 10 a.m. After catheterisation the animals were allowed to run round a room for a short period and almost always faeces were passed at this time. Rarely the faeces were passed in the metabolism cages. The cages were thoroughly cleaned every day. Before the animals could be catheterised easily it was necessary to perform an operation under anaesthesia in which the perineum was split and the vulvar opening thus widened so that a glass catheter could be readily passed. In some instances the animal was catheterised at intervals of about four hours during the day, so as to ascertain the nitrogen excretion during respiratory observations. On these occasions the figures from the analyses are added up to obtain the whole 24 hour results. While the animals were in the metabolism cages the urine was received into bottles through a layer of filter paper, and some crystals of thymol were placed in the bottles to inhibit bacterial growth.

Weight of animals. The animals were weighed carefully before observations in the respiration apparatus. The weighing was generally carried out in the morning after catheterisation and when faeces had been passed and before feeding.

Urine analyses.

Total nitrogen; Kjeldahl's method.

Ammonia; Folin's method.

Urea; Soya bean urease method, as described by Plimmer and Skelton [1914, 1].

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Allantoin was estimated by difference between the figures obtained for urea by Folin's method and by the urease method [Plimmer and Skelton, 1914, 1]. For the estimation of allantoin in diabetic urine the method has been modified by Plimmer and Skelton [1914, 2].

Creatinine; Folin's colorimetric method.

Uric acid; Folin's colorimetric method.

Amino nitrogen; estimation by the difference between the formaldehyde titration and the figure for ammonia by Folin's method.

P_2O_5; Neumann's method as modified by Bayliss and Plimmer.

Acetone and acetoacetic acid; two distillations, the first from acid and the second from alkaline solution, were carried out. The amount of total acetone was estimated by Messinger's iodometric titration. Due precautions were taken to have the tube under the surface of the fluid in the receivers.

Hydroxybutyric acid; estimation by the extraction method of Hurtley. The estimation was discontinued because of the very small quantity present in the dog's urine.

Sugar; the figures obtained by Benedict's method are those given in the tables. The estimations were controlled by the use of Bertrand's method, polarimetric readings, and fermentation in some cases.

Faeces.

Fat; estimation of fat in the dried sample by the Kumagawa-Suto method.

Tissues.

Glycogen; estimation in heart and liver tissue was carried out as described by Cruickshank [1913].

Fat; the method of Kumagawa using Mottram's modification for the removal of unsaponifiable and resinous substances.

Respiration apparatus.

The small respiratory calorimeter devised by Benedict [1912] for animal experimentation was used. The reader is referred to Benedict's articles for a complete description of the apparatus. The construction of the apparatus is essentially the same as that described by Benedict, the only modification being an arrangement for regulating the temperature of the chamber in which the animal is placed. Two incandescent lamps were fixed inside the chamber and the amount of heat given off by them was regulated by a lamp and coil resistance interposed in the circuit outside the box. A thermometer

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reading to tenths of a degree centigrade was placed inside the chamber and a temperature of about 24° C. was maintained during the observations.

Testing the apparatus. The apparatus was tested in the various ways mentioned by Benedict. The parts were first connected to a water manometer and subjected to a pressure of about 100 cm. of water. When the parts were made air-tight separately the whole apparatus was set up and blank determinations made. The weight of the carbon dioxide absorption sets was checked during these blank periods, so as to ensure full absorption of water vapour in the large sulphuric acid bottles. Also the temperature of the box was maintained constant and the level of the spirometer observed for long periods to make sure that the apparatus was air-tight. Finally, an alcohol lamp was burnt inside the chamber and carbon dioxide and oxygen measurements carried out. This test was frequently done in the course of the research and is a very valuable indication of the reliability of results.

Carbon dioxide measurements. The carbon dioxide was estimated by the gain in weight of the soda lime bottle and Williams flask together. The weighings were carried out to 0·01 g. on a large balance capable of weighing ten kilograms. The absorption set was carefully cleaned especially at the coupling joints before every weighing. The blank of determinations showed an error of ± 0·02 to 0·05 g. The absorption set weighed four to five kilos.

The soda lime was made according to the directions given by Benedict and the absorption set was frequently replenished with fresh material. The limit of absorption of the set is about 75 g., but the bottles were replenished before they had increased in weight 50 g.

Oxygen measurements. The oxygen used was that prepared by the electrolytic process and was 99 % pure. It was sent into the apparatus through a gasometer reading to 10 cc., which had been tested for its mechanical error at the ordinary room temperature and for various rates of flow of the gas. This error was:

at rates of 50 to 60 cc. per min.—reading × 0·992.
at rates of 70 to 80 cc. per min.—reading × 0·993.

In many experiments the oxygen was supplied from a small cylinder and the readings of the gasometer were checked by the loss of weight of the cylinder. When the results were satisfactorily checked the readings of the gasometer, corrected for mechanical error, temperature and pressure, were taken, and a large oxygen cylinder could be used.

The spirometer pointer was levelled at the beginning of periods with a fixed pointer and at the end of periods brought to lie along this level again.

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One millimetre rise or fall of the spirometer represents 22 cc. It was found that one-tenth of a degree rise or fall of temperature in the chamber caused a movement of the spirometer corresponding to 30 cc. Care was therefore taken to maintain a constant temperature during periods of observation.

General technique of observations. The periods were usually from 40 to 60 minutes in duration. If the animal became restless towards the end of a period the observation was lengthened in order to avoid as much as possible errors from increases of lung ventilation and from changes of temperature in the chamber. The normal animals show much more restlessness than the diabetics, so that many normal observations were necessary to accustom the animals to the apparatus and thus obtain observations when the animals were quite quiet. The arrangement for recording movement described by Benedict was used and is useful as a rough means of comparing observations.

III. Results.

1. *Respiratory Quotient of Fasting Animals.*

Since the respiratory quotient is a varying ratio depending upon the extent to which protein, carbohydrate and fat are being oxidised in the organism, or being laid down as stores in the depôts of the body, it is very necessary to determine this ratio when the conditions of absorption and utilisation are as uniform as possible. In the fasting state when these conditions are most regular the respiratory quotient can be used as a basis of comparison if certain factors are controlled. The factors which can be controlled are the character of the diet and the time of the observation after a meal. Other factors which must be taken into account are the amounts of carbohydrate and fat stored in the body and available for usage. The 'fasting' respiratory quotients here referred to are observations made at least 18 hours after a meal and under conditions as nearly comparable as possible. In normal animals when meat is fed, the level of the 'fasting' quotient is found to be 0·72 to 0·77 with the usual figure of 0·75. When increasing amounts of meat are fed the quotient is somewhat higher. When large amounts of fat are given the quotient observed is 0·72 to 0·74 in the fasting state, so that whether protein or fat form the main part of the diet of dogs the character of the fasting metabolism appears to be much the same. When starchy foods are fed, the level of the 'fasting' quotient rises to 0·76 to 0·80. In the latter case evidently more carbohydrate material is available for usage or has been stored as glycogen and is taking a uniform part in the

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metabolism, or perhaps some formation of fat from available carbohydrate is taking place. With the 'fasting' respiratory quotients of diabetic animals two points will be observed in the curves and protocols:

(i) the average value is about that observed in the normal combustion of fat, namely, 0·705;

(ii) the respiratory quotient varies within smaller limits than that of normal animals, viz. 0·68 to 0·74.

The lowest fasting quotient of dogs upon a diet rich in fats was 0·690, while the lowest diabetic figure was 0·670. Since the diet of the diabetic

Dog I.

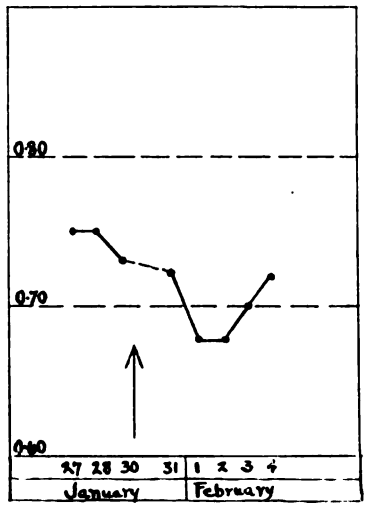

Fig. 1 *a*.

dogs was protein we must compare the 'fasting' quotients with the normal upon protein diet, which shows the diabetic quotient to be at a slightly lower level.

In Fig. 1 (*a* and *b*) the lower level of the 'fasting' quotient of totally depancreatised animals is clearly illustrated, and in most cases this level was lowest soon after the operation and tended to rise later. This rise may be only an apparent one, however, and not due to an increase in the ability to utilise carbohydrate but to a relative decrease in the amount of oxygen taken up. Later on in the course of the severe diabetes produced in totally depancreatised animals, the circulatory weakness and changes in the reaction of the blood evidently combine in causing a diminution in the oxygen intake and thus an apparent rise in the respiratory quotient.

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With the partially depancreatised dogs the course of the fasting quotient varies. Fig. 2 from Dog VII shows that the quotient fell gradually to a low

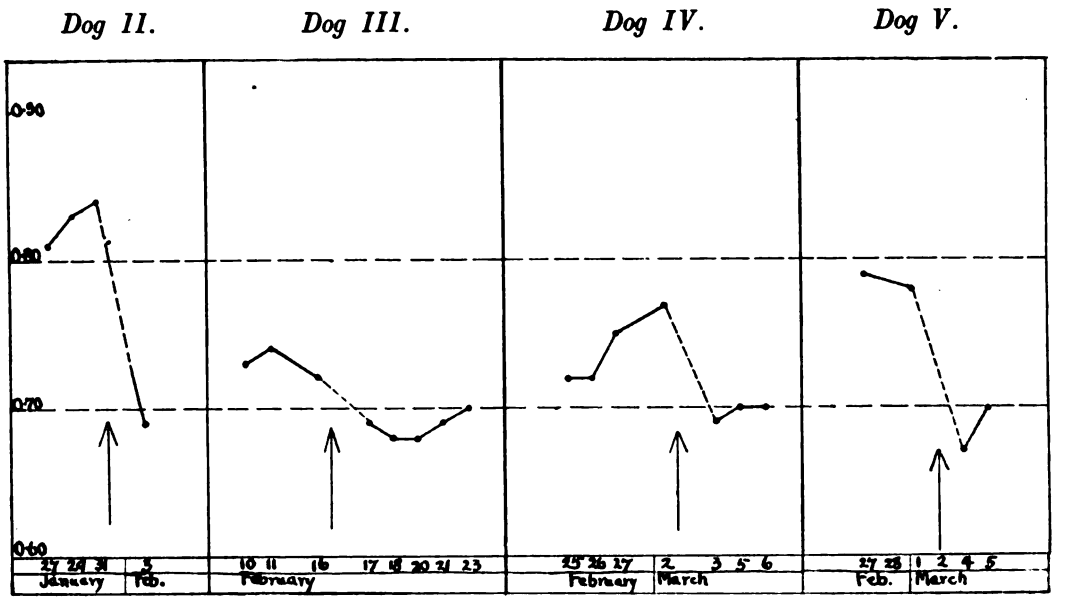

Fig. 1 *b*.

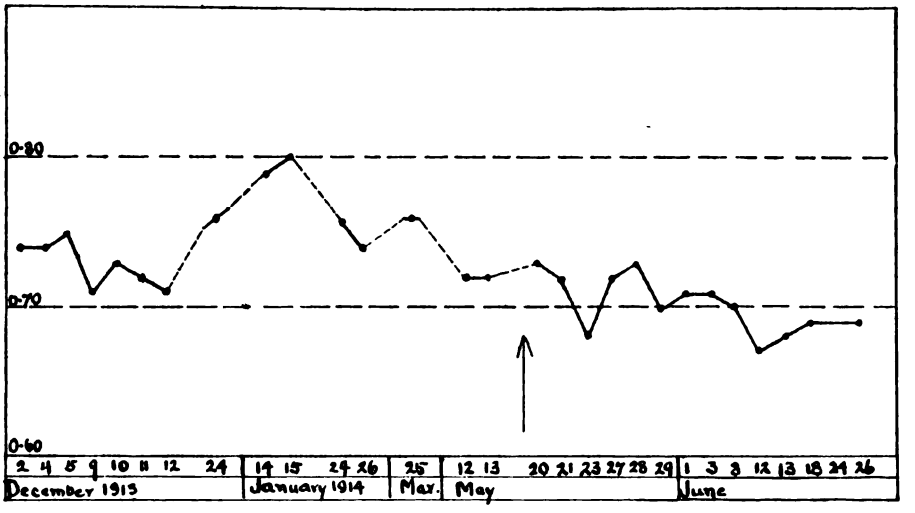

Fig. 2.

level as time went on. The course of the diabetes in this dog was very like that produced by total depancreatisation, except that the animal survived

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longer. With Dog VIII partial depancreatisation produced a very transient and slight glycosuria. Fig. 3 shows that the respiratory quotient reached very low levels soon after the operation and then rose to normal and high levels. After total depancreatisation the course of the quotient was generally higher in Dog VIII than that observed in any of the other diabetic animals, and the general condition of the animal was much better than that of any of the others.

In Figs. 1, 2 and 3, the arrows show when the operations of depancreatisation were performed.

Dog VIII.

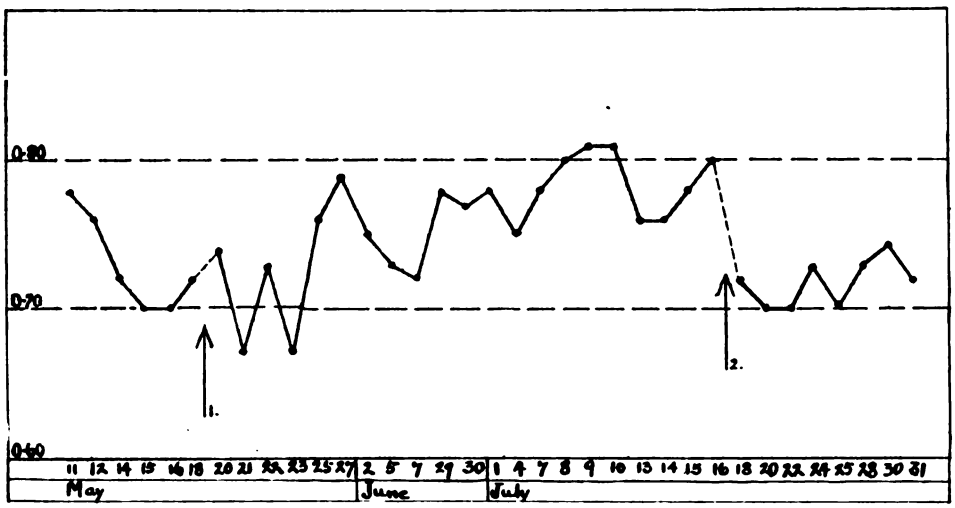

Fig. 3.

2. *Total Metabolism as judged by Oxygen Consumption.*

The figures for the oxygen consumption in the following table (II) are given in cc. per kilo. per hour. They are in every case from observations upon the animals at least 18 hours after feeding and necessarily when the animals showed the greatest degree of rest, and when the figures showed a uniformity in successive periods. Thus the first observation after the animal has been placed in the respiratory chamber has not been used on account of the usual initial restlessness, and the figures for oxygen consumption are an average of successive quiet and uniform periods. The results are therefore comparable with a fair degree of accuracy. Before remarking upon these results it is necessary to mention that there are certain objections to a comparison between

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the total metabolism of normal and diabetic animals based upon the oxygen consumption in cc. per kilo. of animal per hour.

(1) The surface extent of the animal remains approximately constant while the weight of the animal decreases very rapidly when diabetes ensues. There is therefore a relatively greater area where heat loss may occur and one might expect a compensation for this in an increase of metabolism per unit of weight.

(2) The loss in weight in diabetes chiefly concerns water and fat which in the normal condition are in themselves not active consumers of oxygen, while organs and tissues which are constantly and actively engaged in metabolism preserve their normal rate of oxygen consumption. If this occurred, an apparent increase of total metabolism per unit of weight would be the result.

Another consideration in the comparison of total oxygen figures is the fact that the animals become more and more accustomed to and therefore quiet in the apparatus as the observations are continued. This consideration does not apply in the case of the partially depancreatised dogs which were under observation for some time as normal animals. It would apply in the case of the totally depancreatised dogs. Also the depressed state of these diabetic animals must be taken into account. As far as observation and the kymograph records indicate the muscular activity, the diabetic animals were invariably quieter than the normal.

It will be seen from Table II that there is an increase in the oxygen per kilo. per hour amounting to 10–15 % in the diabetic state, taking averages of all normal and diabetic observations. It will be noted that the figures show a steady increase from the time of operation up to the termination. In the last stages it has been remarked that the oxygen intake may decrease markedly owing to circulatory disturbances. The increase in the total metabolism becomes more striking when we remember that the diabetic animals are always quieter than the normal. This increase in the total metabolism we think ought to be regarded as a real increase, not an apparent one, depending upon the two considerations mentioned above. A comparison of the weights and oxygen consumption of Dog VIII in Table II shows that a decrease of weight does not necessarily cause an apparent increase of the total metabolism. This table is divided into six sections so that the figures obtained after both operations and under varying conditions may be compared. It will be noted that a steady decrease in weight occurs in sections II and III without a parallel increase in the oxygen consumption.

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This indicates that the part played by a decrease in the more inert elements of the body or a decrease in weight with the same surface area, in causing an apparent increase in oxygen figures per unit of weight, is at any rate a minor factor. The complications which septic infection of the animals would

TABLE II.

Weight (kilos.) and O_2 consumption (cc. per kilo. per hour).

Dog IV.

Normal Date 1913	Weight	O_2		Diabetic Date	Weight	O_2	
Feb. 27	8·000	541		Mar. 5	7·600	615	
Mar. 2	8·000	517		„ 6	7·300	591	
		529	Average			603	Average

Excess diabetic over normal O_2 consumption = 14 %.

Dog VI.

July 21	7·100	563		July 30	5·900	615	
„ 23	7·000	512					
		537	Average			615	Average

Excess diabetic over normal O_2 consumption = 14 %.

Dog VIII.

May 15	8·250	512	I. Normal	May 20	7·750	525	II. Partially depanc., slight glycosuria
„ 18	8·000	516		„ 21	7·550	518	
				„ 23	7·400	532	
				„ 25	7·200	515	
				„ 27	7·000	438	
		514	Average			505	Average
June 2	6·750	490	III. No glycosuria	July 7	6·650	503	IV. On thyroid, slight glycosuria
„ 5	6·750	431		„ 9	6·500	524	
„ 9	6·750	458		„ 10	6·500	575	
„ 29	6·500	467		„ 13	6·350	535	
„ 30	6·500	466					
July 1	6·500	467					
„ 4	6·600	466					
		463	Average			534	Average
July 14	6·400	473	V. No glycosuria	July 18	6·250	493	VI. Totally depanc. D : N = 3·1
„ 15	6·450	450		„ 20	6·000	524	
„ 16	6·500	431		„ 22	5·800	548	
				„ 24	5·650	553	
				„ 25	5·600	560	Excess period VI over period I = 8 %
				„ 28	5·350	552	
				„ 30	5·200	590	
				„ 31	5·200	623	Period VI over V = 23 %
		451	Average			555	Average

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TABLE II—*Continued*

Dog VII.

1913								
Dec.	5	10·050	543	May	20	8·650	535	Partially depanc. May 18th
„	24	9·600	451	„	21	8·500	532	
1914								
Jan.	14	10·000	432	„	23	8·100	531	
„	24	9·500	545	„	27	7·100	526	
„	26	9·500	520	„	28	7·100	495	
Mar.	25	8·700	451	„	29	7·050	524	
May	12	9·150	488	June	1	6·900	524	
„	13	9·150	479	„	3	6·750	501	
				„	8	6·500	525	
				„	12	6·200	576	
				„	13	6·000	569	
				„	24	5·200	528	
				„	26	5·000	495	
			488 Average				532	Average

Excess diabetic over normal O_2 consumption = 9 %.

Dog I.

1913								
Jan.	27	7·000	563	Jan.	31	6·400	628	
„	28	7·000	579	Feb.	1	6·350	670	
				„	2	6·100	688	
				,	3	5·800	637	
				,	4	5·600	664	
			571 Average				657	Average

Excess diabetic over normal O_2 consumption = 15 %.

Dog III.

Feb.	10	7·600	518	Feb.	17	7·200	575	
„	16	7·500	486	„	21	6·750	595	
				„	23	6·500	594	
			502 Average				588	Average

Excess diabetic over normal O_2 consumption = 16 %.

introduce in comparison of oxygen figures are absent, as the post-mortem examinations revealed no evidence of a generalised septicaemia.

3. *Percentage energy distribution.*

(*a*) *Protein.* From the data obtained in the measurement of the respiratory exchange and estimation of the respiratory quotient it becomes possible to calculate the percentage part of the total metabolism taken by protein, carbohydrate and fat. This calculation is made by subtracting from the total figures the amounts of carbon dioxide and oxygen corresponding to the protein metabolised and estimating the respiratory ratio of the remainder,

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which indicates quantitatively the extent to which carbohydrate and fat are being used. An example may be given:

Dog VII. Dec. 3, 1913.

	CO_2	O_2	R.Q.
Fasting 23 hrs. Starch diet	4160	5418 →	0·760
Nitrogen, g. per hour 0·110	537	664	
	3623	4754 →	0·762

In this example we find that the protein portion amounts to 12 % of the total and the quotient of the remainder shows that the carbohydrate percentage is 17·0 and the fat 71·0 %. The figures for one hour are used. The nitrogen excretion is calculated in this example from the 24 hours analysis and the figures for CO_2 and O_2 corresponding to protein are taken from Schumburg and Zuntz's table quoted by von Noorden [1907]. It is evident that, in order to obtain a correct value for the protein percentage, the nitrogen excretion should be estimated during the respiratory observation. An estimate based upon the 24 hours nitrogen in the above example is not so liable to error as it would be if a protein rich diet were being fed. In the latter case such a nitrogen figure would give too high a percentage for protein during a fasting period and too low in observations after feeding. In the normal dogs 15 to 20 % was found to be the usual protein percentage of the fasting metabolism when meat was fed, and where direct determinations of

TABLE III. *Percentage energy distribution. Fasting observations. Normal.*

No. of animal	Date	Hours after feeding	Diet	N	Carbo-hydrate	Fat	% energy distribution: Pro-tein	Carbo-hydrate	Fat	
	1914									
III	Feb. 10	18	P.b.	—	—	—	15·0	6·0	79·0	Protein p.c. assumed
	„ 16	17	Meat	4·0	—	—	22·0	0·0	78·0	
VI	July 23	23	Meat	8·0	—	—	15·0	12·0	73·0	„
	1913									
VII	Dec. 3	23	P.b.	4·5	120·0	—	12·0	17·0	71·0	
	„ 4	18	P.b.	1·6	60·0	—	12·0	8·0	80·0	
	„ 5	19	P.b.	3·3	135·0	—	13·0	13·0	74·0	
	„ 24	18	Mixed	11·0	70·0	—	15·0	14·0	70·0	„
	1914									
	Jan. 15	18	Mixed	4·7	60·0	—	15·0	30·0	55·0	„
	Mar. 25	24	C. and Pal.	6·6	—	40·0	15·0	15·0	70·0	„
	May 9	17	Meat	10·0	—	—	15·0	10·0	75·0	„
	„ 14	22	Meat	10·0	—	—	15·0	0·0	85·0	„
VIII	„ 18	24	Meat	8·0	—	—	15·0	3·0	82·0	„

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nitrogen excretion have not been made, this figure (15 %) has been assumed. In the tables it is indicated where the protein percentage has been directly calculated and where it has been assumed.

Table III contains a summary of these calculations from the respiratory and nitrogen figures of normal fasting animals. In Table IV will be found the observations made after feeding protein and fat. The results obtained after administration of sugars will be found in the section dealing with carbohydrate.

TABLE IV. *Percentage energy distribution in digesting animals. Normal.*

No. of animal	Date	Hours after feeding	Diet	N	Carbohydrate	Fat	R. Q.	% energy dist. to Protein	Carbohydrate	Fat
	1914									
III	Feb. 12	4 to 6	Meat	4·0	—	—	0·806	44·0	19·0	37·0
	„ 13	5 to 7	Meat	4·0	—	—	0·798	41·0	18·0	41·0
	1913									
VII	Dec. 13	2–3	Meat	8·0	—	—	0·818	79·0	10·0	11·0
	„ 15	2–3	Meat	10·0	—	—	0·802	90·0	1·3	8·7
	„ 15	6	Meat	10·0	—	—	0·786	77·0	3·0	20·0
	„ 22	3–5	Meat	4·0	—	—	0·820	68·0	17·0	15·0
	1914									
	Feb. 2	3	Butter	—	—	50·0	0·733	9·5	7·0	84·0
	„ 9	3	P.b.	3·0	60·0	—	0·900	17·0	75·0	8·0
	„ 13	3	P.b.	3·0	60·0	—	0·855	20·0	60·0	20·0
	„ 24	5–7	Meat	8·0	—	—	0·802	62·0	11·0	27·0
	Mar. 5	7	Meat Erept.	9·0	—	—	0·803	74·0	7·5	18·5
	„ 11	3	C. and Pal.	—	—	—	0·767	17·0	13·0	70·0
	„ 18	4	C. and Pal.	—	—	—	0·744	29·0	0·0	71·0
	„ 19	1	C. and C.L.O.	—	—	—	0·750	33·0	0·0	67·0
	„ 20	3	C. and C.L.O.	—	—	—	0·707	10·0	0·0	90·0
	„ 23	4	C. and Pal.	—	—	—	0·730	20·0	0·0	80·0
VIII	June 22	2	Bread and Meat	—	—	—	0·921	34·0	66·0	0·0
	July 2	3	P.b.	—	—	—	0·861	30·0	43·0	27·0

When the animals are diabetic calculations of the percentage energy distribution become more complicated, owing to the fact that part of the protein which normally makes up the protein quota of the total metabolism is lost to the organism as excreted sugar. Beside this sugar formation from protein which we attempt to allow for in the calculations of percentage energy distribution, other processes may occur, such for example as the formation of sugar from fat or of the acetone bodies, which may still further complicate such energy estimations. The calculations, therefore, even after allowing for sugar formation from protein, are more a rough indication than a quantitative index, because of complexities for which one cannot allow. We have, however, thought that some indication would be afforded of the

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character and extent of the carbohydrate and fat portions. In order, therefore, to get some idea of the energy distribution, a side calculation has been made allowing for sugar formation from protein based upon the observed D : N ratio. If one allows for the sugar formation from protein, as in the following example, the respiratory quotient of the remaining protein carbon dioxide and oxygen can be easily calculated. As the dextrose-nitrogen ratio increases from 2·6 to 4·0 the respiratory quotient decreases from 0·725 to 0·620. It can be readily seen, therefore, how much a perversion of protein metabolism with, say, a D : N ratio of 3·8 would contribute to the lowering of the respiratory quotient of diabetics. Such an example is:

Dog IV. Mar. 3, 1914.

Fasting 48 hours. Total N 5·10. D : N 2·9	CO_2	O_2	
Nitrogen per hour 0·210	1026	1268	Protein to sugar correction
Glucose „ 0·60	444	444	
	582	824	

	CO_2	O_2	R.Q.
Total figures observed	3643	5146 →	0·700
Protein minus glucose	582	824	
	3061	4322 →	0·707

In this example the protein percentage is 16 % and the remaining 84 % has a quotient indicating fat combustion. A number of such calculations have been made and are given in the tables. The percentage part in the total metabolism played by protein is increased in diabetes according to our observations. The protein percentage ranges from 16 to 32 % on the first and second days after depancreatisation, with an average of 25 %.

Table VII (p. 200) contains a summary of the figures for percentage energy distribution in the diabetic dogs in the fasting and digesting state. This table contains a number of observations which may be compared with those in Tables III and IV. In the digestion of a protein meal the level of the respiratory quotient is much lower in the diabetic animals than in the normal, and the allowance for sugar formation from protein in most cases indicates that the energy is distributed to protein and fat and that the oxidation of the fat portion is complete.

In Table V are given the results of calculations of the percentage energy distribution in diabetic dogs on the early days after operation. The protein portion is reckoned on the total 24 hours nitrogen. It will be noted that this protein percentage varies greatly, but the usual figure is distinctly higher

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than the normal percentage when no protein has been given for periods of 48 to 60 hours. The excessively high figure in the case of Dog VII after partial depancreatisation is surprising. In this dog there was an extremely slight glycosuria and in the calculation no allowance is made for sugar form-

TABLE V.

No. of dog	Depanc.	Days after operation	Prot.	Carb.	Fat
I	Total	1st	32·0	4·0	64·0
		2nd	30·0	0·0	70·0*
III	Total	1st	24·0	0·0	76·0*
IV	Total	1st	16·0	0·0	84·0
V	Total	2nd	18·0	0·0	82·0*
VI	Total	2nd	21·0	10·0	69·0
VII	Partial	2nd	30·0	10·0	60·0
		3rd	24·0	7·0	69·0
VIII	Partial	2nd	62·0	0·0	38·0*
	Total	1st	52·0	2·0	46·0
	Total	2nd	19·0	2·0	79·0

ation from protein, since practically no sugar was excreted, but the results indicated the probability of such formation and also that the sugar, if formed, was unutilised since the quotient of the remainder after deducting the protein portion is far below that of the combustion of fat. The full figures of this observation will be given:

Dog VII. May 20. 72 *hours since food was given.*

	CO_2 1 hour	O_2 1 hour	R.Q.
Total N 9·5 g. Total sugar excretion 2·25	2889	3894 →	0·742
Protein 1 hour N 0·40	1954	2415	
Carbohydrate and fat ..	935	1479 →	0·632

Protein 62 %. Fat 38 % (incomplete?).

With this dog it will be noted that two months later, on the first day after the total depancreatisation, the protein figure is again very high, but when one allows for the sugar formation from protein on the basis of the observed D : N ratio, one sees that the combustion of the fat portion is complete.

Dog VII. July 18. 44 *hours since any food was given.*

	CO_2	O_2	CO_2	O_2	R.Q.
Total N 9·8 g. N 0·408	1994	2463	2198	3084 →	0·712
D : N 2·8, Gl. 1·14	847	847	1147	1616	
	1147	1616	1051	1468 →	0·715

Protein 52·0. Carbohydrate 2·0. Fat 46·0.

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It therefore appears very probable that in the first instance sugar was being formed from protein, that this sugar was not being utilised to any great extent and also that it was not being excreted. We obtained no striking evidence of incomplete combustion of fat in an increase of acetone bodies excreted, which indicates that the conclusions are justified.

(*b*) *Fat.* The percentage energy distribution to fat in the normal animals in a fasting condition can be seen from the results in Table VIII to be uniformly high. Feeding the animals upon fat alone was not carried out with success, but with large amounts of fat fed with protein the fat takes a predominant part in the total metabolism (Table IV, p. 186) with a resultant lowering of the respiratory quotient. We obtained no evidence of incomplete combustion of fat in the normal animals. Great interest centres around the question of the combustion of fat in diabetes. In Table V the figures for fat marked (*) are cases in which the combustion of fat was apparently incomplete as the remainder quotient after deducting for protein was lower than the normal fat quotient. We find no parallelism between this apparent incomplete combustion of fat and the excretion of acetone bodies. The latter increase steadily throughout the course of the diabetes and frequently show fluctuations which cannot be correlated with any marked change in the respiratory quotient. The course of excretion of acetone bodies seems to bear a closer relation, though not very close, to the protein breakdown. Also in the cases in which fat was fed to the animals the excretion of fat in the faeces never indicated a deficient absorption of the fat fed. The fats, therefore, as far as these observations upon dogs indicate, are apparently well dealt with by the diabetic organism. In some cases evidence of incomplete combustion of fat appears. In the greater number of cases the low respiratory quotient of diabetic dogs appears to be caused, not so much by a perversion of the fat metabolism as by the disturbance of the normal combustion of protein.

(*c*) *Carbohydrate.* The extent to which carbohydrate is oxidised in diabetic as compared with normal dogs is, of course, the question which is the most important to us at present. It is necessary, therefore, to find out what percentage part of the total metabolism is taken by carbohydrate in the normal animal under various conditions.

In the normal animal it is obvious that the amount of sugar used in the oxidations will depend firstly upon the supply available. Thus in the fasting animal the percentage distribution to carbohydrate may vary from 0 to 30 % depending upon the diet, whether rich or poor in carbohydrate, and the carbohydrate storage in the tissues. Table III (p. 185) contains the figures

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calculated as described above from a series of normal fasting observations.

Dog III.

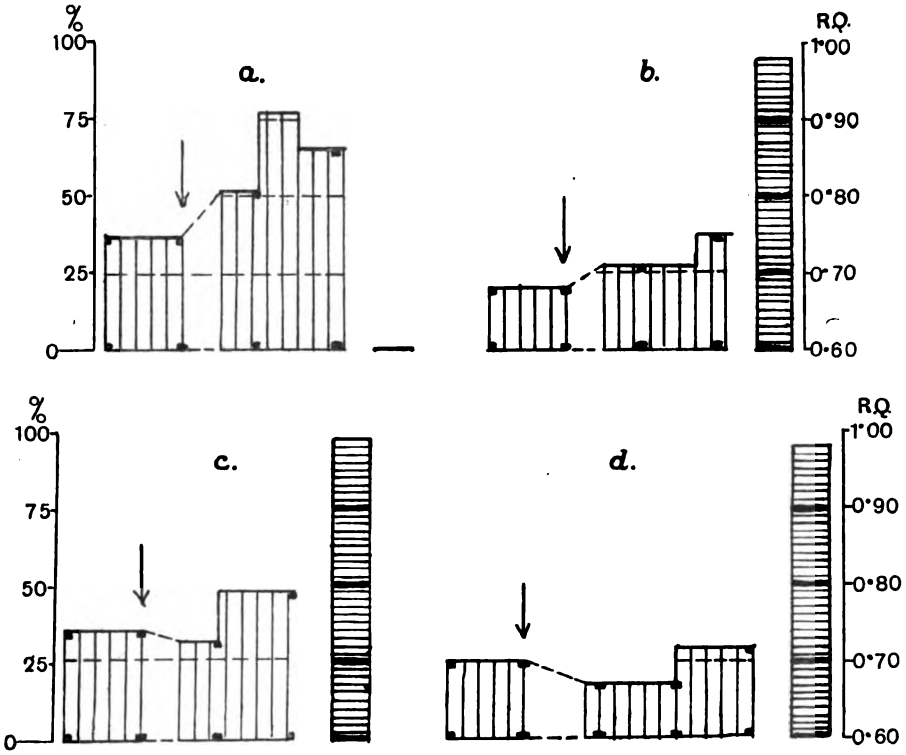

Fig. 4.

Dog IV.

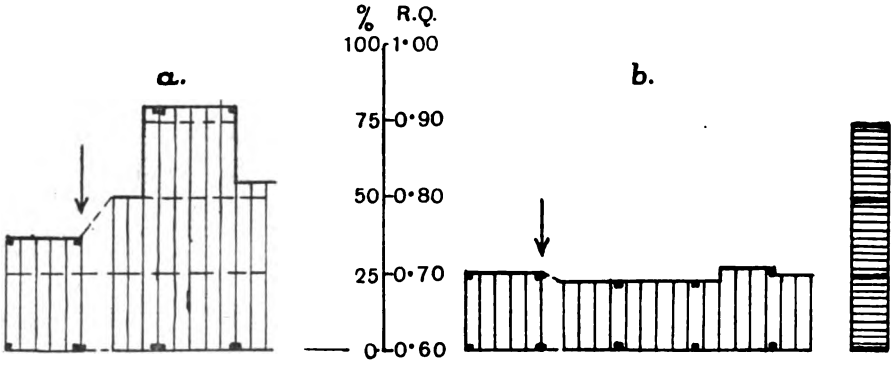

Fig. 5.

When glucose or fructose is given to normal animals the utilisation is shown by a marked rise of the respiratory quotient. Within an hour after

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Dog VII.

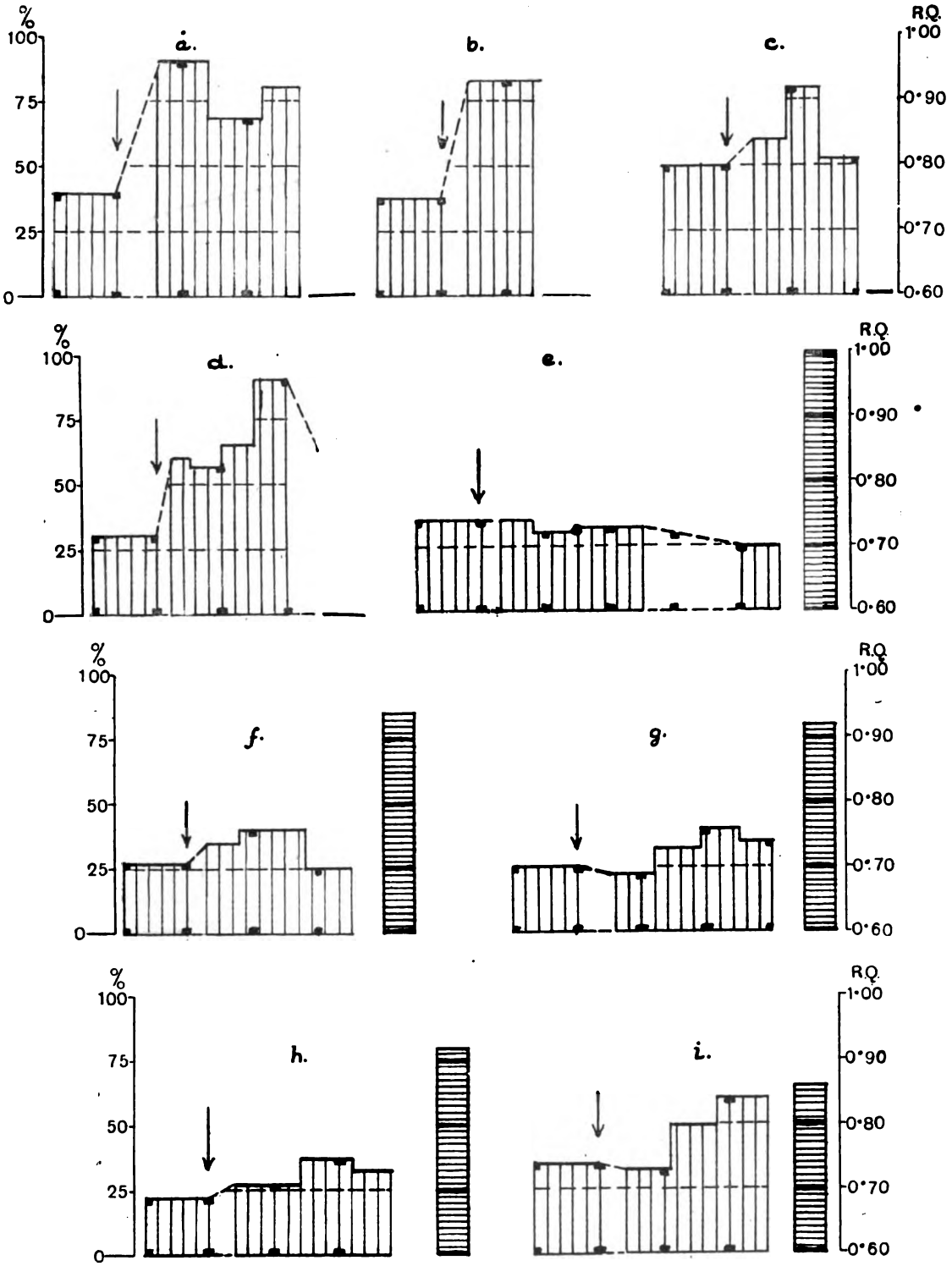

Fig. 6.

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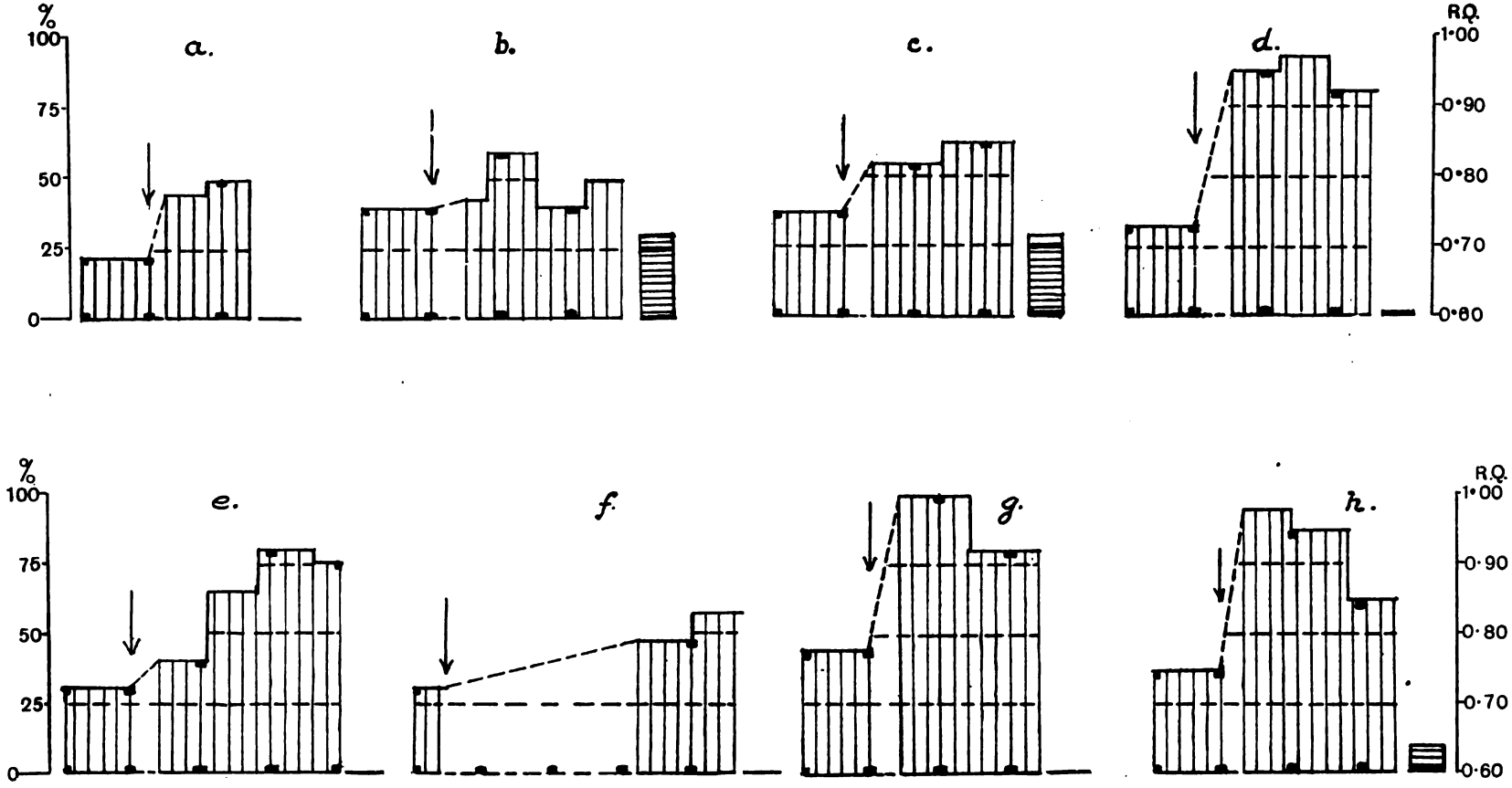
Dog VIII.



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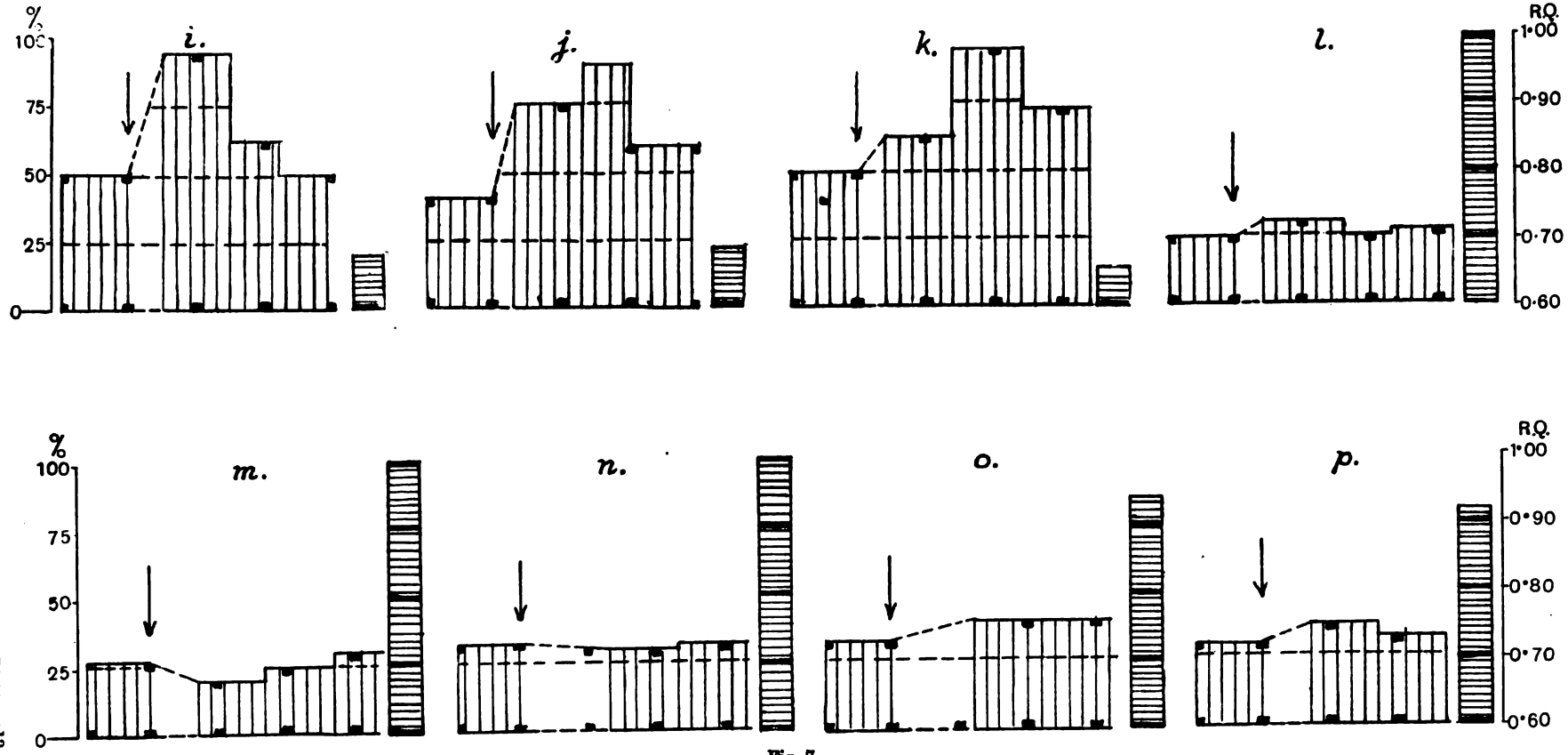

Fig. 7.

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the sugar has been taken the quotient has risen to 0·90 to 1·00 and for three or more hours remains between 0·80 and 0·90, gradually returning to its original level. If calculations are made of the percentage energy distribution, the carbohydrate portion increases up to 80 to 90 % (see Table VI). The following is a protocol of an observation before and after the giving of glucose:

Dog IV. Feb. 27, 1914.

Period No.	Duration mins.	CO_2 cc. per min.	O_2 cc. per min.	R.Q.	Protein	Carbo-hydrate	Fat
I	50	54·1	72·2	0·749	15·0[1]	10·0	75·0
30 g. glucose given 20 mins. before II period.							
II	20	85·2	106·0	0·803	15·0	27·0	68·0
III	40	84·6	92·2	0·917	15·0	67·0	18·0
IV	30	77·7	84·5	0·919	15·0	70·0	15·0
V	30	71·5	87·6	0·816	15·0	35·0	50·0

[1] Assuming protein to be 15 %.

With the doses of glucose given (20 to 40 g.) no sugar appeared in the urine of normal dogs. In the normal rise of the quotient after giving sugar the minute figures for carbon dioxide and oxygen both increase, but the amount of the carbon dioxide increases far more than the oxygen intake. The rise in the respiratory quotient is due to an increase in the output of CO_2.

In Figs. 4, 5, 6 and 7 (pp. 190–193) a series of observations before and after the giving of sugar are plotted as curves. The curves hatched vertically represent the levels of the respiratory quotient during the successive periods. Five of the vertical lines represent a duration of one hour. The arrow marks the time at which the sugar was given. The second part of the curve is hatched horizontally and shows the percentage of the sugar which was recovered from the urine, over and above the amount of sugar corresponding to the nitrogen excreted on the basis of the D : N ratio of the day before and after. The protocols corresponding to Figs. 4, 5, 6, 7 will be found in Table VI. In the normal animal it is seen from these results that the rise of the respiratory quotient after sugar is a well-marked one. If we compare these results with the effect of giving sugar to the diabetic animals, two important points are brought out:

(i) The rise of the respiratory quotient in the diabetic animals is diminished in all cases, and frequently fails to appear.

(ii) The level of the quotient after giving sugar corresponds very closely to the percentage excretion of the sugar given. If all the sugar is excreted the quotient shows little or no rise, and if only a slight excretion occurs, as in the case of Dog VIII, the increase in the respiratory quotient approaches the normal.

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TABLE VI. *Respiratory quotient, minute figures for CO_2 and O_2 and percentage energy distribution before and after sugar administration.*

No. of animal	Date	Period No.	Duration min.	Sugar	CO_2 cc. p. m.	O_2 cc. p. m.	R.Q.	% energy distribution: Protein	Carbohydrate	Fat	Notes
	1914										
III	Feb. 11	I	60		66·75	89·5	0·745	15·0	9·0	76·0	Normal.
	a.			Gluc. 30 g.							
		II	30		76·0	94·0	0·806	—	—	—	
		III	30		67·26	75·9	0·908	15·0	65·0	20·0	
		IV	35		68·5	79·43	0·862	—	—	—	Depancreatised Feb. 16.
	Feb. 18	I	60		57·86	85·5	0·680	24·5	0·0	75·5	D : N = 3·2.
	b.			Gluc. 30							
		II	70		55·5	78·3	0·713	26·8	3·2	70·0	
		III	20		59·8	79·2	0·754	—	—	—	Sugar excreted = 95 %.
	Feb. 19	I	40		54·2	73·0	0·740	25·0	11·0	64·0	D : N = 3·4.
	c.			Gluc. 20							
		II	20		59·5	82·1	0·724	26·0	20·0	54·0	
		III	30		52·6	66·6	0·789				
		IV	30		77·5	98·1	0·790	—	—	—	Sugar excreted = 95 %. Dog very restless.
	Feb. 21	I	60		48·05	67·9	0·699	21·0	4·0	75·0	D : N = 3·4.
	d.			Gluc. 20							
		II	40		49·3	74·4	0·672	20·0	0·0	80·0	
		III	30		48·5	72·3	0·671				
		IV	60		58·15	81·3	0·723	18·0	6·5	75·5	Sugar excreted = 95 %.
IV	Feb. 27	I	50		66·85	89·1	0·750	—	—	—	
	a.			Gluc. 30							
		II	20		85·2	106·0	0·803	—	—	—	
		III	40		84·6	92·2	0·917	—	—	—	
		IV	30		77·7	84·5	0·920	—	—	—	
		V	30		71·5	87·6	0·820	—	—	—	Depancreatised Mar. 2.
	Mar. 6	I	60		51·05	72·4	0·705	15·0	2·0	83·0	D : N = 3·8.
	b.			Gluc. 30							
		II	43		58·4	84·0	0·696	—	—	—	
		III	41		57·3	83·7	0·685	—	—	—	
		IV	40		56·0	81·3	0·688	13·0	0·0	87·0	
		V	40		55·0	76·7	0·716	—	—	—	
		VI	32		55·97	79·03	0·708	15·0	2·0	83·0	Sugar recovered 75 %.
	1913										
VII	Dec. 3	I	45		69·34	90·3	0·760	12·0	17·0	71·0	Normal.
	a.			Gluc. 30							
		II	45		83·55	86·95	0·96	12·7	82·0	5·3	
		III	45		67·98	77·98	0·87	—	—	—	
		IV	45		73·24	79·95	0·92	—	—	—	
	Dec. 5	I	45		68·50	91·0	0·750	13·0	13·0	74·0	
	b.			Fruct. 30							
		II	60		95·55	102·98	0·930	11·0	73·0	16·0	

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TABLE VI—*Continued*

No. of animal	Date	Period No.	Duration min.	Sugar	CO_2 cc. p. m.	O_2 cc. p. m.	R.Q.	% energy distribution: Protein	Carbohydrate	Fat	Notes
	1914										
VII	Jan. 15	I	30		64·3	79·77	0·807	15·0	30·0	55·0	
	c.			Gluc. 10							
		II	30		64·8	77·3	0·840	—	—	—	
		III	30		73·8	80·4	0·920	15·0	70·0	15·0	
		IV	30		68·0	84·5	0·805	—	—	—	
	May 14	I	40		52·8	73·0	0·720	15·0	0·0	85·0	
	d.			Gluc. 20							
		II	20		74·8	89·35	0·837	—	—	—	
		III	30		68·7	83·5	0·822	—	—	—	
		IV	30		65·5	75·86	0·860	—	—	—	
		V	30		70·94	73·4	0·966	15·0	85·0	0·0	Partially depancreatised May 18.
	May 28	I	60		46·1	61·9	0·743	32·0	13·0	55·0	D : N = 2·9.
	e.			Gluc. 20							
		II	33		49·0	65·8	0·744	—	—	—	
		III	40		51·77	71·7	0·720	—	—	—	
		IV	60		55·5	75·5	0·730	28·0	10·0	62·0	Sugar excreted 100 %.
		V	32		46·0	66·4	0·691	30·0	0·0	70·0	4 hrs. after the sugar.
	June 3	I	60		40·55	56·4	0·718	14·0	0·0	86·0	D : N = 3·1.
	f.			Gluc. 20							
		II	30		39·0	53·0	0·736	—	—	—	
		III	60		47·9	63·17	0·758	17·0	12·0	71·0	
		IV	40		44·52	63·55	0·700	15·0	0·0	85·0	85 % of sugar excreted.
	June 8	I	60		39·9	56·94	0·700	14·0	4·0	82·0	D : N = 3·7.
	g.			Fruct. 20							
		II	40		52·3	76·2	0·686	20·0	5·0	75·0	
		III	43		46·16	62·8	0·734				
		IV	37		44·13	58·1	0·759	23·0	20·0	57·0	
		V	30		47·3	64·1	0·738				Sugar excreted 80 %.
	June 13	I	50		38·6	56·90	0·678	20·0	0·0	80·0	D : N = 3·4.
	h.			Gluc. 20							
		II	60		37·33	52·16	0·715	2·0	5·0	93·0	
		III	45		43·06	57·4	0·749	2·0	15·0	83·0	
		IV	35		42·0	57·0	0·736	—	—	—	Sugar excreted 80 %.
	June 24	I	60		34·35	46·32	0·741	20·0	17·0	63·0	D : N = 3·8.
	i.			Gluc. 20							
		II	40		53·8	73·9	0·727	—	—	—	
		III	40		46·17	58·0	0·796	—	—	—	
		IV	40		39·05	46·6	0·837	23·0	40·0	37·0	Sugar excreted 65 %.
	June 18	I	80		54·81	77·4	0·707	40·0	7·0	53·0	D : N = 3·5. Intravenous injection.
				Gluc. 20							
		II	35		54·0	77·4	0·698	—	—	—	
		III	35		53·8	76·1	0·706	—	—	—	
		IV	30		55·9	79·0	0·708	39·0	5·0	56·0	

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TABLE VI—*Continued*

No. of animal	Date 1914	Period No.	Duration min.	Sugar	CO_2 cc. p. m.	O_2 cc. p. m.	R.Q.	% energy distribution: Protein	Carbohydrate	Fat	Notes
VIII	May 16	I	30		53·26	77·1	0·690	10·0	0·0	90·0	Normal.
	a.			Gluc. 10							
		II	35		62·1	79·3	0·782	—	—	—	
		III	35		55·7	70·0	0·796	10·0	30·0	60·0	
	May 25	I	60		46·9	61·8	0·758	15·0	14·0	71·0	Partial depancreatisation on May 18.
	b.			Gluc. 20							Slight glycosuria.
		II	20		52·4	67·8	0·773	—	—	—	
		III	40		48·25	57·25	0·842				
		IV	40		50·9	66·5	0·765	15·0	28·0	57·0	Total sugar exc. = 11·0 g.
		V	30		54·66	68·4	0·798				
	May 29	I	60		43·75	58·4	0·748	15·0	10·0	75·0	
	c.			Gluc. 20							
		II	60		48·66	59·36	0·820	—	—	—	
		III	60		52·08	61·5	0·846	15·0	43·0	42·0	Total sugar exc. = 5·9 g.
	June 5	I	35		35·6	48·57	0·733	15·0	6·0	79·0	No glycosuria.
	d.			Fruct. 20							
		II	42		54·05	56·9	0·949	—	—	—	
		III	40		50·4	51·7	0·974	15·0	85·0	0·0	
		IV	40		51·9	56·3	0·921	—	—	—	No sugar excreted.
	June 9	I	40		37·3	51·6	0·723	15·0	0·0	85·0	
	e.			Gluc. 20							
		II	40		42·75	56·0	0·763	—	—	—	
		III	40		50·4	58·2	0·864	—	—	—	
		IV	45		49·66	53·8	0·922	15·0	70·0	15·0	
		V	20		49·6	54·8	0·904	—	—	—	No sugar excreted.
	July 1	I	60		39·3	50·66	0·777	15·0	20·0	65·0	
	g.			Gluc. 20							
		II	60		54·9	52·85	1·03	15·0	85·0	0·0	No sugar excreted.
		III	60		49·95	54·3	0·920				
	July 4	I	45		38·44	51·33	0·749	15·0	9·0	76·0	Thyroid administered.
	h.			Gluc. 20							
		II	40		52·4	53·25	0·984	10·0	90·0	0·0	
		III	50		62·6	66·2	0·946	—	—	—	
		IV	40		46·3	54·37	0·851	—	—	—	Total sugar exc. = 2·1 g.
	July 9	I	60		47·57	58·95	0·806	15·0	26·0	59·0	
	i.			Gluc. 20							
		II	60		65·66	67·08	0·978	15·0	85·0	0·0	
		III	40		52·8	62·17	0·849	—	—	—	
		IV	40		49·75	62·17	0·800	15·0	25·0	60·0	Total sugar exc. = 4·3 g.
	July 14	I	60		38·55	50·47	0·764	15·0	16·0	69·0	
	j.			Gluc. 20							
		II	60		44·66	49·65	0·900	—	—	—	
		III	40		49·0	51·1	0·958	15·0	80·0	5·0	
		IV	50		49·36	58·68	0·841	—	—	—	Total sugar exc. = 4·8 g.

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TABLE VI—*Continued*

No. of animal	Date	Period No.	Duration min.	Sugar	CO_2 cc. p. m.	O_2 cc. p. m.	R.Q.	% energy distribution Protein	Carbohydrate	Fat	Notes
	1914										
VIII	July 16	I	60		37·65	46·7	0·806	15·0	29·0	56·0	Thyroid stopped.
	k.			Gluc. 20							
		II	60		39·6	46·5	0·852	—	—	—	
		III	60		44·77	45·73	0·979	10·0	90·0	0·0	
		IV	60		41·9	47·2	0·887	—	—	—	Total sugar exc. 3·3 g. Totally depancreatised, July 17.
	July 20	I	60		37·39	53·4	0·700	36·0	0·0	64·0	D : N = 3·0.
	l.			Gluc. 20							
		II	71		39·3	54·8	0·717				
		III	40		40·35	57·45	0·702	35·0	1·0	64·0	Sugar excreted 100 %.
		IV	50		38·36	53·92	0·711				
	July 22	I	60		37·63	53·01	0·709	20·0	4·0	76·0	D : N = 3·1.
	m.			Gluc. 20							
		II	60		38·25	55·93	0·684	—	—	—	
		III	60		40·2	57·03	0·705	20·0	0·0	80·0	
		IV	40		38·8	53·4	0·720	—	—	—	Sugar excreted 100 %.
	July 24	I	60		38·06	52·13	0·730	20·0	10·0	70·0	D : N = 3·1.
	n.			Gluc. 20							
		II	62		38·0	52·64	0·721	—	—	—	
		III	60		39·16	53·66	0·730	20·0	7·0	73·0	Sugar excreted 100 %.
	July 28	I	60		36·13	49·26	0·733	20·0	12·0	68·0	D : N = 3·1.
	o.			Fruct. 20							
		II	60		38·76	50·8	0·763	20·0	20·0	60·0	Sugar excreted 85 %.
		III	60		39·1	51·53	0·758				
	July 31	I	50		38·88	54·0	0·720	15·0	7·0	78·0	D : N = 3·1.
	p.			Gluc. 20							
		II	60		44·02	58·73	0·749	14·0	13·0	73·0	Sugar recovered 80 %.
		III	60		42·33	57·95	0·730				

When the respiratory quotient shows no rise, as for instance with Dog IV *b*, Fig. 5, the minute figures for carbon dioxide and oxygen will be found to show a parallel increase; see protocol IV *b* in Table VI.

In some of the totally depancreatised dogs in the later stages, it was observed several times that the respiratory quotient might rise noticeably, simulating the elevation after giving sugar. The cause of this rise was found to be a diminution in the oxygen intake with a very constant carbon dioxide output. The following is an instance:

Dog I.

Feb. 4th, 1913.		CO_2	O_2	R.Q.
Period I.	40 mins.	45·67	62·72	0·728
Given 15 g. glucose				
Period II.	30 mins.	45·60	60·2	0·750
Period III.	42 mins.	46·70	54·0	0·863
Feb. 5th. No sugar given				
Period I.	30 mins.	39·6	63·6	0·624
Period II.	45 mins.	39·4	46·7	0·844
Period III.	40 mins.	39·7	53·5	0·742

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This animal was in a very bad condition when these observations were made. This condition may lead to confusing results and the quotient is of no value unless the minute figures are analysed. The variations in the taking up of oxygen may be due to a periodic type of breathing, but we regard the duration of the observation as long enough to equalise any such effect, and think that the blood changes must be responsible for the continual decrease in the taking up of oxygen observed. The respiration rate has been observed to vary from 15 to 43 per minute without any irregularity in the oxygen intake—no measurement of the depth of respiration was made. Several instances such as the above have been observed when the animals showed great circulatory and muscular feebleness, and were so quiet in the apparatus that the possibility of errors in the oxygen measurement is excluded.

As is seen in Figs. 5, 6 and 7 the rise of the respiratory quotient caused by sugar is absent in observations shortly after depancreatisation and later may occur in a very diminished form. In the totally depancreatised Dog III some evidence of sugar utilisation is present shortly after the operation, but the last observation (Fig. 4) indicates practically no utilisation of sugar. Dog IV when diabetic showed no indication that sugar was being used (Fig. 5 *b*). The series of observations shown in Fig. 7 on Dog VIII gives a very good comparison of the partially and totally depancreatised state. In this figure *b* and *c* are made shortly after the partial depancreatisation and show less marked increase of the R. Q. than the succeeding observations when practically none of the sugar was recoverable from the urine. The dog apparently recovered its normal power of utilising glucose. The observations *l*, *m*, *n*, *o* and *p*, after the remnant of pancreas was removed, show very little evidence of sugar utilisation. Dog VII shortly after partial depancreatisation gave no indication of sugar utilisation, as is seen in Fig. 6 *e*. Later the graft apparently established its disturbed circulatory conditions and a return of the power of oxidising sugar is evident from the rise of the respiratory quotient (Fig. 6 *h*, *i*).

The percentage energy distribution to carbohydrate has been seen to vary in the normal fasting condition and after the giving of sugar to constitute a large part, 80 to 90 %, of the total metabolism. In such calculations in the fasting state of diabetic animals the figures, after allowance for sugar formation from protein, indicate that a small part is taken by carbohydrate in some cases. The results will be found in Table VII. Whether this is an apparent or real utilisation of carbohydrate can be seen to depend upon the accuracy with which the sugar formation from protein can be estimated from the D : N ratio observed. Thus, if too great an amount of sugar is taken to correspond with

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TABLE VII.

Respiratory quotients and percentage energy distribution in diabetic animals.

No. of animal	Date	Hours after	Diet	N	Carbo-hydrate	Fat	R.Q.	% energy dist. Protein	Carbo-hydrate	Fat	D/N	Notes
I	Jan. 31	40	Meat	12·0	—	—	0·717	32·2(1)	4·0	64·0	3·1	
	Feb. 1	64	Meat	12·0	—	—	0·691	30·0	0·0	**70·0**(2)	3·0	
	„ 2	24	Meat	8·0	—	—	0·688	29·0	0·0	**71·0**	2·7	
	„ 3	20	Meat	4·0*	—	—	0·708	24·0	3·0	73·0	3·2	* Intake less—dog vomiting.
	„ 4	18	Meat	4·0*	—	—	0·720	9·0	9·0	82·0	3·2	
III	Feb. 17	41	Meat	4·0	—	—	0·692	24·0	0·0	**76·0**	2·7	
	„ 18	3	M. and P.	4·0	—	—	0·680	24·5	0·0	**75·5**	3·2	
	„ 19	3	M. and P.	4·0	—	—	0·740	25·0	11·0	64·0	3·4	
	„ 20	7	M. and P.	4·0	—	—	0·680	41·0	0·0	59·0	3·8	
	„ 21	18	M. and P.	4·0	—	—	0·690	21·0	4·0	75·0	3·4	
	„ 23	19	M. and P	4·0	—	—	0·705	22·0	3·0	75·0	3·4	
IV	Mar. 3	48	Meat	4·0	—	—	0·700	16·0	0·0	84·0	2·9	
	„ 4	5	M. and P.	5·5	—	—	0·691	21·0	0·0	79·0	3·5	
	„ 6	20	M. and P.	4·0	—	—	0·705	15·0	2·0	83·0	3·8	
V	Mar. 4	67	M. and P.b.	8·0	50·0	—	0·677	18·0	0·0	**82·0**	3·0	
	„ 5	4	M. and P.	4·0	—	—	0·700	36·0	0·0	64·0	3·0	
	„ 7	36	M. and P.	4·0†	—	—	0·670	10·0	0·0	**90·0**	4·0	† Dog vomiting.
VI	July 29	60	Meat	8·0	40·0†	—	0·731	21·0	10·0	69·0	3·1	Fructose day before.
VII	May 20	72	Meat	8·0	—	—	0·733	30·0	10·0	60·0	2·6	Milk 100 cc. 19 hrs. before.
	„ 21	24	Milk	1·0	10·0	6·0	0·725	24·0	7·0	69·0	2·6	
	„ 23	18	M. and P.	8·5	—	—	0·676	34·0	0·0	**66·0**	3·2	
	„ 27	17	M. and P.	8·5	20·0	—	0·715	42·0	3·0	55·0	2·8	Glucose day before.
	„ 28	18	M. and P.	8·5	—	—	0·743	32·0	13·0	55·0	2·9	
	„ 29	18	M. and P.	7·5	20·0	—	0·693	36·0	0·0	**64·0**	2·9	Glucose day before.

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	June 1	24	M. and P.	7·5	—	—	0·706	30·0	0·0	70·0	2·8	
	„ 2	3	M. and P.	7·5	—	—	0·693	43·0	0·0	**57·0**	2·8	
	„ 3	22	M. and P.	7·5	—	—	0·710	14·0 N.O.	0·0	86·0	3·1	
	„ 8	24	M. and P.	9·5	—	—	0·700	14·0 N.O.	4·0	82·0	3·7	
	„ 13	18	M. and P.	10·0	—	—	0·693	20·0 N.O.	0·0	80·0	3·4	
	„ 18	3	Cas. and P.	6·0	—	—	0·707	40·0 N.O.	7·0	53·0	3·5	
	„ 24	18	Gel. and P.	6·0	—	—	0·741	20·0 N.O.	17·0	63·0	3·8	
	„ 26	24	Cas. and P.	6·0	—	—	0·696	19·0	0·0	81·0	3·7	
VIII	May 20	72	Meat. Milk 100 cc. day before	8·0	—	—	0·742	62·0(1)	0·0	**38·0**	—	Total sugar 2·25.
	„ 25	24	M. and P.	5·5	—	—	0·758	36·0	6·0	58·0	—	Total sugar 11·0.
	„ 29	24	M. and P.	5·5	—	—	0·748	27·0	9·5	63·5	—	Total sugar 5·9.
	June 22	2	Bread and Meat	6·5	100·0	—	0·921	34·0	61·0	5·0	—	No glycosuria.
	„ 29	18	P.b.	5·0	60·0	—	0·780	34·0	14·0	52·0	—	„ „
	„ 30	18	Meat and P.b.	7·0	60·0	—	0·770	31·0	12·0	57·0	—	„ „
	July 2	3	P.b. and Meat	7·0	80·0	—	0·861	30·0	43·0	27·0	—	
	„ 7	24	P.b. and Meat. Thyroid	7·0	60·0	—	0·785	28·0	18·0	54·0	—	
	„ 8	19	„ „ „	7·0	60·0	—	0·809	29·0	26·0	45·0	—	
	„ 9	18	„ „ „	7·0	60·0	—	0·806	35·0	22·0	43·0	—	
	„ 18	44	M. and P.	11·0	20·0	—	0·712	19·0	2·0	79·0	2·8	
	„ 25	18	„ „	11·8	20·0	—	0·710	20·0*	2·0	78·0	3·1	
	„ 27	4	„ „	5·0	—	—	0·780	33·0	28·0	39·0	3·1	
	„ 29	3	„ „	5·9	20·0	—	0·740	44·0	17·0	39·0	3·1	
	„ 30	19	„ „	5·9	20·0	—	0·745	16·0(1)	15·0	69·0	3·1	
	„ 31	18	„ „	11·8	—	—	0·720	15·0(1)	7·0	78·0	3·1	

(1) Protein metabolism has been calculated from 24 hours nitrogen excretion, except where marked * when the percentage has been assumed, and N.O. where the nitrogen excretion has been determined during the respiratory observation.

(2) Figures in black type denote that the calculation indicated an incomplete combustion of fat or other disturbance associated with abnormally low quotient.

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the nitrogen excretion, deduction of the carbon dioxide and oxygen figures corresponding to protein minus sugar will indicate an apparent combustion of carbohydrate. In the majority of cases this amount of carbohydrate apparently utilised is quite small and frequently after sugar is given does not show any increase whatever, which is difficult of explanation if sugar is really being utilised.

4. *Results of Urine Analyses.*

Table VIII contains the results of the urine analyses as well as the total intake of food, balance sheets for nitrogen and phosphate, and general notes as to the condition of the animals.

(*a*) P_2O_5. It was thought that the increase of excreted phosphate indicated the tissue break-down in the diabetic animals. When a balance is estimated over a period, however, there is very little discrepancy between the intake and output. Dog VII showed a loss of 2·5 g. and Dog VIII a gain of 0·5 g. during the period.

(*b*) *Acetone bodies.* In Table VIII the figures for acetone represent the total acetone preformed and present as acetoacetic acid in the urine. The excretion of acetone bodies starts on an upward course soon after depancreatisation and shows many fluctuations from day to day with the general increase as the condition becomes more severe. We can find nothing definite to account for the considerable rise in the amount of acetone bodies in Dog VII on June 9 (Table VIII). 10 g. of sodium bicarbonate were administered on that day, but since on other occasions this does not cause a marked rise in acetone excretion, it may be due to something else. The question as to the chief source of the acetone bodies will be dealt with later in the report.

(*c*) *Sugar.* The sugar excreted in the diabetic urine was almost always dextro-rotatory and apparently glucose. When glucose was given to the diabetic dogs it was excreted in large amount. If the animals were catheterised after three to four hour periods, the rate of excretion of the sugar given could be ascertained. If we compare the sugar and nitrogen excretion after giving sugar (see Table IX) we see that the sugar is excreted in this case in large proportion during the first three hours after administration. On June 8 (Table IX) 20 g. fructose was administered and a small proportion of the sugar excreted was laevo-rotatory. On June 18 the animal received a protein meal before the first four hour period and the results illustrate the fact that the sugar is excreted before the nitrogen of the protein given.

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(*d*) *Nitrogen excretion.* The total nitrogen balance shows a marked loss of nitrogen from the body in the early stages of diabetes. Later this loss becomes smaller and with a high protein intake equilibrium may be reached. Since the nitrogen loss is so marked in the early stages and the proportion of

TABLE IX. *Dog VII. Excretion of sugar and nitrogen after giving glucose and fructose.*

Date	No. of Period	Dur'n hours	Total glucose	Total nitrogen	D/N	N g. per hour	Notes
1914							
June 3	I	4	0·325	0·470	0·70	0·117	
	II	2·5	15·0	0·390	38·4	0·156	Given glucose 20 g. at beginning of period II.
	III	17·5	24·5	6·80	3·6	0·388	Meat and pancreas = 7·5 g. N at beginning of period III.
		24	39·8	7·66	5·1	—	Totals.
		24	38·5	7·14	5·3	—	Control on 24 hrs sample.
June 8	I	3	1·20	0·49	2·4	0·163	
	II	3	10·30[1]	0·80	13·0	0·266	Fructose 20 g. at beginning of period II.
	III	18	43·5	9·9	4·4	0·55	
		24	55·0	11·19	4·9	—	Totals.
		24	51·0	—	—	—	Control on 24 hrs sample. Glucose 48·0 Fructose 3·0
June 13	I	2	0·10	0·42	0·24	0·210	
	II	3	12·9	0·056	212·0	0·019	Glucose 20 g.
	III	19	35·7	9·13	3·9	0·480	Caseinogen and pancreas = 9·6 g. N at beginning of period III.
		24	48·7	9·61	5·0	—	Totals.
June 18	I	4	14·4	2·2	6·5	0·55	Caseinogen and pancreas = 6·0 g. N at beginning of period I.
	II	4	7·35	2·1	3·5	0·525	Intravenous injection 1·5 g. glucose at beginning of period II.
	III	16	21·8	7·0	3·1	0·44	Caseinogen and pancreas = 6·9 g. N at beginning of period III.
		24	43·5	11·3	3·8	—	Totals.
June 24	I	4	3·7	0·70	5·2	0·175	
	II	3	14·0	0·68	20·0	0·226	Glucose 20 g. at beginning of period II.
	III	17	41·7	11·82	3·5	0·695	Gelatin and pancreas = 12·9 g. N at beginning of period III.
		24	59·4	13·20	4·5	—	Totals.

[1] Dextrose 8·42. Fructose 1·88. Meat and panc. = 10·0 gm. N at beginning of period III.

sugar to nitrogen relatively small, and later increasing, the impression is given that the protein break-down in the earlier stages is resulting in the formation of sugar which accumulates in the blood to no purpose and overflows through the kidneys when a certain level is reached. This indication

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has been mentioned above in connection with the increased protein metabolism in the partially depancreatised Dog VIII when practically no sugar was excreted. The total nitrogen excretion in the case of Dogs I and V is large directly after the total extirpation and decreases to a very small amount. This is accounted for by the fact that the animals on the fourth day after the operation vomited most of the food taken and the nitrogen excretion therefore indicates the break-down of the animal's own tissues.

(*e*) *Ammonia.* In Table VIII the ammonia is given both as g. and % of nitrogen. When expressed as percentage of total nitrogen the ammonia figure increases somewhat after total depancreatisation in the case of Dog III. In Dog VII the ammonia nitrogen percentage of the total nitrogen shows a slight increase when diabetes was produced. The increase is from 2 % to 3 % above the normal figure.

(*f*) *Urea.* As can be seen from Table VIII the percentage of urea nitrogen to total nitrogen is slightly lower in the diabetic animals than in the normal fed upon the same diet. In Dog VII, when normal and fed on meat and pancreas, the urea percentage was 85 to 90 % and when the animal was diabetic the percentage was slightly lower—80 to 87 %. Dog III before depancreatisation had a urea percentage of 90 % and after the operation 82 to 86 %.

(*g*) *Allantoin.* The average normal excretion of allantoin in the dog when meat is fed amounts to 0·2 to 0·5 g. N, or 2·4 % of the total nitrogen (see Table VIII). In the presence of sugar the allantoin estimation is upset unless special precautions are taken [Plimmer and Skelton, 1914, 2]. The allantoin figures for the diabetic dogs are of no value except in the case of Dog VIII. The allantoin nitrogen in this case increases up to 1·16 g. in the diabetic state and the percentage of the total nitrogen is about 10 %.

(*h*) *Uric acid.* As will be seen from the analyses figures for Dogs III and I, uric acid appears in the diabetic urine in estimable amounts and shows an increase as the diabetes progresses. In the normal dog the uric acid excretion is very small.

5. *Faeces.*

Analyses of the faeces for total nitrogen and fat and phosphate were carried out by Dr Cruickshank. The total nitrogen and P_2O_5 figures were used in estimating the balances to which reference has been made. The fat elimination in the faeces was generally small in normal and diabetic animals and the fats fed were evidently absorbed without difficulty from the intestine.

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6. *Post-mortem Findings.*

General. The animals all showed great emaciation. The abdominal organs were examined, particularly the site of the pancreas on the duodenum. In the total depancreatisations the duodenum was removed and suspicious tissue examined histologically. No remnants of pancreatic tissue were found after the total extirpations. Portions of the heart and liver and in some cases suprarenals and thyroid were removed immediately after death.

Dog I. No signs of suppuration about wound or in the abdominal cavity. No remnants of pancreas present on the duodenum. Heart muscle flabby and pale. Liver pale. Suprarenals removed and histological appearance found to be normal.

Dog III. Some adhesions about the duodenal borders where pancreas had been removed. No pancreatic tissue found. No signs of suppuration or inflammation about wound or abdomen. Lymphatic nodes were enlarged, but this was noted at the operation. Artery walls very friable. This was noted also on many occasions by Evans, Patterson and Starling in their experiments on the heart-lung preparation of diabetic animals.

Dog VII. Some adhesions about the duodenum. Abdominal wound quite healed and closed. Graft was found to the right of the middle line under the skin of abdomen. It was hard, white and about one cm.^{2} in area. Blood supply evidently came to graft through a fold of omentum which was adherent to inner side of the abdominal wound. No remnants of pancreatic tissue in abdomen.

Histological appearance of graft. The tissue shows some, but not very marked evidence of degeneration. Acinous tissue in places looks quite normal. No islets of Langerhans could be identified with certainty.

Dog VIII. Adhesions between duodenum and colon and liver. Liver yellowish. Some small masses of tissue were removed from the duodenum and on histological examination were found to be masses of scar and lymphatic tissue—no pancreas remains found. No signs of suppuration about wound or abdomen.

Histological examination. Remnant of pancreas removed from the duodenum at operation II was found to show very little sign of degeneration. Acinous tissue was normal with some vacuolisation. Islets of Langerhans could be distinguished.

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7. *Analysis of Organs for Glycogen and Fat.* (*Dr Cruickshank.*)

The glycogen estimations which have been carried out agree with the general results of Cruickshank's former investigation. The great increase of glycogen in the heart in the case of the partially depancreatised Dog VII is of interest, but more estimations are necessary before any conclusion can be

No. of animal	Organ	Glycogen %	Fat %	
VII	Heart	1·40	1·89	
	Liver	0·10	6·10	
VIII	Heart	0·924	2·6	
	Liver	0·025	10·7	
VI	Heart	0·50	2·37	
	Liver	0·20	5·84	
Normal	Heart	0·49	1·50	Average figures for normal animals.
	Liver	4·0 to 7·0	2–3 %	

drawn. The results have indicated that the heart muscle retains its power of adding to its glycogen store in diabetes and, perhaps because of the increased available amount of sugar, has actually a greater glycogen reserve than the normal. The increase in the percentage of fat in both cardiac and hepatic tissue is an evidence of the fat mobilisation in diabetes. A further analysis of the mobilisation of fat in diabetes would be of value and, it is hoped, will shortly be undertaken.

IV. Discussion and Summary.

Total Metabolism in Experimental Diabetes.

The results show a definite increase of total metabolism in pancreatic diabetes. This increase corresponds with the severity of the usual symptoms. In Dog VIII when partially depancreatised no increase was observed and the glycosuria was slight and transitory. In Dog VII partial removal of the pancreas resulted in a marked glycosuria and the average increase in the total metabolism is 9 %. In the totally depancreatised animals the increase was from 14 to 23 % and the type of diabetes very severe. Murlin and Kramer [1913] found an increase of metabolism amounting to 42 % in a depancreatised dog. Our figures represent an average throughout the course of the diabetes and the increase per cent. is found to rise during the diabetic period (Dog VIII) from 9 % to 38 %. The increase of total metabolism parallels the severity of the diabetes. Benedict and Joslin [1910, 1912] in their studies of diabetes in man calculated that the increase of

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metabolism was from 15 % to 20 %. Lusk and others objected to this conclusion on the ground that the controls were unsuitable for comparison

Dog VII.

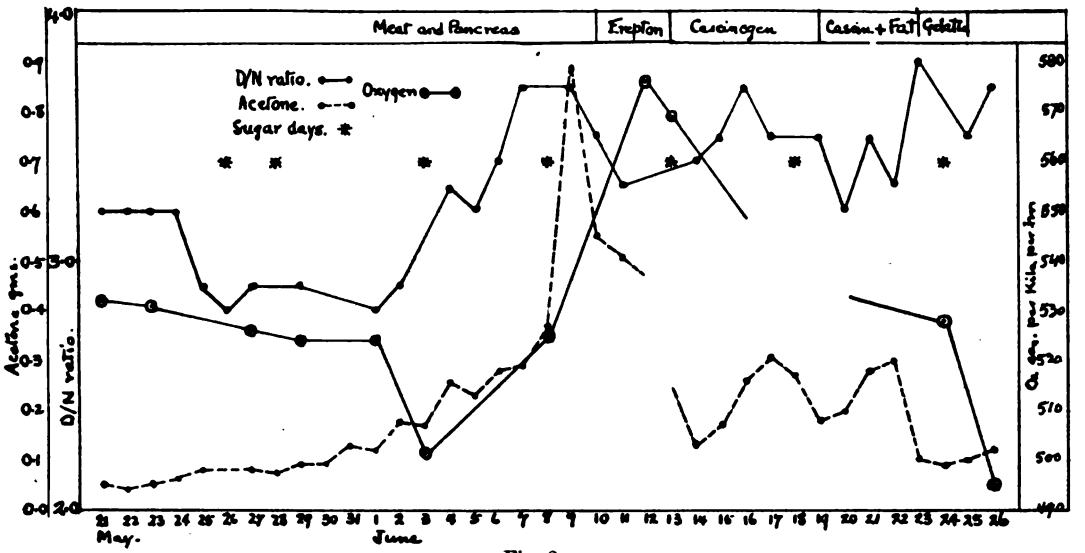

Fig. 8.

Dog VIII.

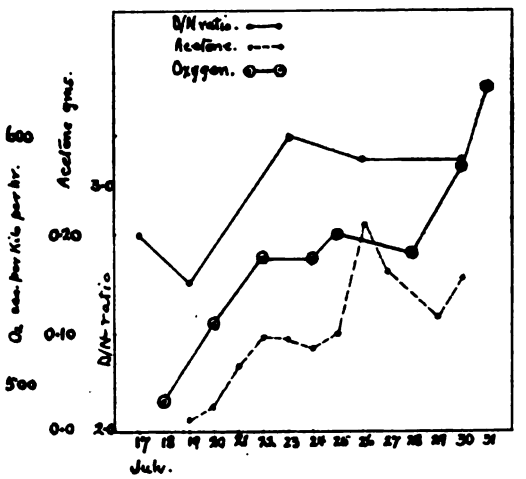

Fig. 9.

with the diabetics because of differences in size and weight. More recently, however, Benedict and Joslin in their report on cases of severe diabetes have

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substantiated their former finding. Their later normal controls are strictly comparable in the matter of dimensions and the authors find the increase in diabetes more marked than previously stated. Taken as an isolated fact this increase in metabolism cannot be used as a deciding argument in favour of either the over-production or the non-utilisation theory. The fact that the total metabolism increases more and more during the course of the diabetes might be taken to indicate that this phenomenon is secondary to some other process. It is interesting, therefore, to find out what parallelism exists between the increase of total metabolism and other evidences of disturbance. In Figs. 8 and 9 the D : N ratio, the total acetone excretion, and the oxygen consumption in cc. per kilo. per hour of the diabetic Dogs VII and VIII, have been charted. The acetone excretion is subject to some fluctuation but a certain amount of parallelism exists between the course of the oxygen consumption and the acetone excretion. The three curves in Fig. 9 show a corresponding rise. In the case of Dog III when diabetic these three curves also show a parallel rise. There seems to be a relationship between the degree of disturbance of the protein metabolism and the oxygen consumption. As far as one can judge from the calculations of energy distribution an increase in fat metabolism or evidence of incomplete combustion of fat does not seem to bear a relation to the increase in total metabolism. Some interesting observations of Benedict and co-workers show that, in the normal human, an acidosis induced by a carbohydrate-free diet is accompanied by an increase in the total metabolism. It is apparently difficult to induce an acidosis in normal dogs and in some experiments with fat feeding (Dog VII, Table VIII) observations upon the acetone excretion and respiratory exchange did not show any marked variation. The part played by sugar usage in relation to the changes in total metabolism in diabetic dogs is an important consideration. The evidence as to the power to oxidise sugar, which is exhibited by animals during the course of the diabetes, consists in the level of the respiratory quotient and the degree of rise after sugar is given. From this evidence it has been seen that the degree of utilisation is sometimes less shortly after depancreatisation than later on. This is evident from the series of curves in Fig. 6 from the partially depancreatised Dog VII. In this case a limited degree of sugar usage occurred after three weeks. The course of the total metabolism does not seem to bear a close relation to the evidences of sugar usage. In the totally depancreatised animals the diminished utilisation of sugar is evident from the beginning of the diabetic period, while the oxygen consumption shows a steady increase throughout.

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This comparison of the course of the two processes would seem to indicate that the disturbance of carbohydrate metabolism is the primary process and the gradual increase in total metabolism an evidence of a cumulative disturbance in protein and perhaps fat metabolism or an attempt to make up for the deficiency in the energy exchanges caused by the imperfect utilisation of sugar.

The increase in protein metabolism in diabetes has been noted by many investigators. The protein break-down is shown by the marked loss of nitrogen to the body especially in the earlier days of diabetes. In our experiments calculation of the energy distribution to protein after allowance has been made for sugar formation shows that the increase is 5 % to 10 % over the normal protein value in most cases. The actual protein break-down in the fasting condition has been shown to be enormously increased in pancreatic diabetes [Falta, Grote and Staehelin, 1907] and in phloridzin glycosuria [Reilly, Nolan and Lusk, 1898]. The protein destruction as evidenced by the nitrogen elimination shows an enormous increase, while the actual part taken by protein in the oxidations is only slightly increased. This process may be illustrated by the figures from two observations upon a dog in the normal and diabetic condition after the ingestion of meat.

(*a*) *Dog III. Normal.*

	CO_2	O_2	R.Q.
Feb. 12, 1914. Wt. 7·600	3768	4677 →	0·806 (observed)
N in g. per hr. = 0·340 =	1662	2053	
	2106	2624 →	0·802

in which the protein percentage is 44·0 %, carbohydrate 19 %, fat 37 %.

(*b*) *Dog III. Diabetic* 4 *days. D : N ratio* 3·8.

	CO_2	O_2	R.Q.
Feb. 20, 1914. Wt. 6·800	3132	4624 →	0·680 (observed)

	CO_2	O_2	CO_2	O_2	R.Q.
N in g. per hr. = 0·595 =	2907	3592	1227	1912	
Sugar from protein = 2·26 g. =	1680	1680	1905	2712 →	0·705
	1227	1912			

Protein 41·0 %. Carbohydrate 0·0 %. Fat 59·0 %.

Both observations are six hours after meat containing 4·0 g. nitrogen had been eaten. Hourly figures are given.

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Comparison of these two observations will show that, while the protein percentage of the total oxygen distribution is practically the same in both cases, the nitrogen excretion, and therefore the protein destruction, is greatly increased.

In the protein break-down which occurs in diabetes the form in which the nitrogen is excreted might give some indication of the disturbance. Expressed in percentage of the total nitrogen the urea nitrogen tends to diminish and the ammonia to increase, and the increase in ammonia nitrogen corresponds generally with the increased excretion of acetone bodies. The balance thus maintained by the urea and ammonia nitrogen is an expression of the process by which the organism attempts to counteract the increasing acidosis, as has been generally recognised in cases of human diabetes. The destruction of tissue protein is shown also in the increase of the purine metabolism in diabetes. The allantoin excretion in one dog where the estimation in the presence of sugar is satisfactory formed up to one gram of the total nitrogen or about 10 %. The allantoin nitrogen in the normal animal under the same conditions of diet amounts to 0·5 g., or 3 % of the total nitrogen. The excretion of uric acid in the normal dog is very small and showed a definite increase in diabetes. The phosphate excretion in diabetes is slightly greater than the intake, but the phosphate balance shows such an inconsiderable loss that it cannot be taken as evidence of tissue destruction.

The protein destruction, as shown by the great loss of nitrogen to the body, starts soon after the removal of the pancreas, and the intake has to be greatly increased before a balance is reached. In this increase of the protein metabolism in the first few days of diabetes the amount of nitrogen excreted per hour is equal to that observed after a protein meal in the normal condition, but the respiratory exchanges do not show the increase in total metabolism which would occur were the protein fully oxidised. If, on the basis of the D : N ratio, we subtract the energy equivalent of the sugar formed from protein, the energy distribution to protein in the total metabolism is found to be somewhat increased. These facts indicate that the well recognised break-down of protein is closely associated with the power of the tissues to oxidise carbohydrate.

The question as to what significance can be attached to the steadily increasing excretion of acetone bodies in experimental diabetes is of interest. The possible sources of these intermediary products are the proteins or fats, and some indication has been sought for as to whether they originate mainly from alteration in the protein or fat metabolism. On a carbohydrate-free

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diet normal dogs do not show any marked increase of acetone excretion. With feeding of fats to Dog VII no such increase was observed. As soon as pancreatic diabetes is established the acetone excretion increases steadily and shows a parallel course to the D : N ratio and the increase in total metabolism. If the acetone bodies had their source in incomplete fat combustion one would expect the respiratory quotient to fall in relation to the rise in acetone excretion. This, however, does not appear to be the case. The energy distribution calculations on days when the acetone excretion is highest do not show any marked indication of incomplete fat oxidation. The total acetone excretion in the diabetic dogs is not large and the production of a small amount might not influence the level of the respiratory quotient. The general course of the acetone excretion bears a closer relation to the protein metabolism. Variations in the total nitrogen are usually accompanied by parallel variations in the total acetone. In view of the fact that amino-acids, such as leucine and tyrosine, have been observed to produce acetoacetic acid, while many of the other amino-acids of protein produce sugar in diabetes, the influence of feeding different proteins and erepton was studied in Dog VII. In this dog the acetone excretion reached its highest level upon the standard diet of horse meat and pancreas. Upon erepton the acetone excretion was high and fell somewhat with caseinogen feeding. When gelatin was fed the acetone excretion fell considerably while the D : N ratio rose. We have therefore some indication that the acetone excretion depends on the character of the proteins fed. In the case of gelatin, which contains a larger proportion of sugar-producing amino-acids, the results are well marked. The most important source of the acetone bodies in the diabetic metabolism of dogs appears to be protein, and the increasing disturbance in the protein metabolism is indicated by the excretion of the acetone bodies as well as by the D : N ratio.

Utilisation of Sugar in Diabetes.

The generally low level of the respiratory quotient in diabetes is one of the chief arguments in favour of the non-utilisation theory. It might be argued, however, that in diabetes other processes which require much oxygen and yield less carbon dioxide obscure an oxidation of carbohydrate which is as effective as in the normal condition. The processes which would contribute to give very low respiratory quotients are the following:

(*a*) The formation of sugar from protein. Magnus Levy has calculated that the respiratory quotient yielded by protein after the full amount of

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sugar formed is allowed for, is 0·615. In our calculations with a D : N ratio ranging from 2·7 to 3·8 the respiratory quotient of protein decreases from 0·715 to 0·640.

(*b*) The formation of acetone bodies from protein.

(*c*) The formation of acetone bodies from fat.

(*d*) The formation of sugar from fat.

We have attempted in our calculations to allow for the formation of sugar from protein. The formation of acetone bodies has not been taken into account in the calculations, because the small amounts excreted indicate that the part played by this process in the low respiratory quotient is a minor one. The respiratory quotient of the formation of sugar from fat is very low. The figures cannot be corrected for the formation of sugar from fat because there are no data to go upon. If this process occurred to any extent in diabetes the respiratory quotient should reach much lower values than those generally observed.

After making allowance for the first of the factors mentioned above, we still find very little evidence of sugar utilisation in the diabetic dogs, and the apparent utilisation when it occurs may be due to error in the use of the D : N ratio in the calculation.

The diminished rise or constant level of the respiratory quotient after giving glucose to diabetic dogs is also evidence of the inability of the tissues to oxidise glucose. The course of the respiratory quotient agrees with the percentage of sugar recovered from the urine. When a rise of the respiratory quotient does occur, after giving sugar to totally depancreatised animals it is in most cases small and within the limit of possible error. The disturbance of sugar utilisation is apparent shortly after depancreatisation and does not appear to increase with the severity of the diabetes. The giving of sugar later in the course of the diabetes resulted in a limited utilisation in the case of Dog VIII. The rise in the respiratory quotient after fructose (Table VI, protocol *o*) indicated a utilisation of 0·30 g. of sugar per hour during the observations. After glucose (protocol *p*) the utilisation was 0·25 g. per hour in the same dog three days later. In the partially depancreatised Dog VII the course of the sugar utilisation is similar, but the ability to oxidise sugar returned to a greater degree. In this latter case the return of the sugar usage can be explained by the pancreas tissue present, which evidently became more functional after a time had elapsed. An explanation of the apparent partial recovery in the power of using sugar in Dog VIII is harder to find. Remnants of pancreas tissue were carefully searched for at the

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post-mortem without result. It may be that the tissues elaborate to some degree some other way of dealing with carbohydrate than that which exists when the pancreas is intact. Another possibility is that the increasing number of organisms in the intestinal tract and elsewhere account for the oxidation of sugar which occurs. The general condition of the animals has usually corresponded with the ability to use carbohydrate; thus Dog VIII after total depancreatisation was in much better condition than any of the other diabetic dogs. The results, therefore, indicate that removal of the pancreas in dogs causes a serious disturbance in the carbohydrate metabolism, in which the power of oxidising glucose is, for a time, completely lost and, while this may return to a small degree, it is never again observed to approach the normal in extent.

The recent work of Verzar [1914] on the question of sugar utilisation in diabetes has also given support to the non-utilisation theory. Verzar finds that, when sugar is injected into the circulation of diabetic animals, the normal rise of the respiratory quotient does not occur, but that this evidence of the loss of sugar utilisation is obtained only some days after the removal of the pancreas. In our experiments the effect of sugar giving was tried for the first time about the third or fourth day after depancreatisation, and only in the case of Dog III did we obtain evidence of a limited sugar utilisation two days after the operation. The results obtained from the injection of glucose and fructose therefore lead to the same conclusions as our findings in regard to the sugar utilisation in diabetes.

Summary.

1. In experimental diabetes an increase of the total metabolism occurs, amounting to 15 to 20 % on an average, and increasing with the severity of the condition.

2. The rise of the respiratory quotient which normally occurs after the giving of glucose or fructose is greatly diminished or is absent after the total removal of the pancreas, indicating that complete oxidation of sugar does not occur.

3. The increase in the excretion of acetone bodies parallels the increase in the D : N ratio and the total metabolism, suggesting that the phenomena are expressions of a similar disturbance of protein metabolism which is secondary to the impairment of sugar utilisation.

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tal Nitrogen							
Amino	Creatin.	Undet. N	Temp.	Pulse rate	Resp. rate	Weight	Notes
—	—	—	—	—	—	7·0	Depancreatised.
0·4	0·7	5·9	37·6	—	17	6·4	Bright and active.
0·9	0·6	8·8	—	—	—	6·350	
0·9	0·6	—	—	—	—	6·100	Depressed and weak.
1·0	0·7	11·5	—	—	—	5·800	Very depressed and weak; coughing.
2·1	1·0	—	—	—	—	5·600	Very weak; coughing; vomiting; diarrhoea; will not eat.
—	—	—	35·0	100	—	5·200	Extreme weakness; died under anaesthetic.
—	—	—	—	—	—	[illegible]	
—	—	—	—	—	—	—	
—	—	—	—	—	—	7·000	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	Very well; abdominal skin incision healed.
—	—	—	—	—	—	—	
—	—	—	—	—	—	6·750	P_2O_5 intake 11·0; P_2O_5 output 10·5.
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	6·750	
—	—	—	—	—	—	—	Removed from metabolism cage.
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	Removed from cage.
—	—	—	—	—	—	6·500	
—	—	—	—	—	—	—	
—	—	—	—	—	—	6·500	
—	—	—	37·8	74–80	—	—	In very good condition.
—	—	—	—	—	—	—	Thyroid administration: 1 g.
—	—	—	—	—	—	6·600	1·0
—	—	—	—	—	—	—	1·5
—	—	—	—	—	—	—	1·5
—	—	—	—	—	—	6·650	2·0
—	—	—	39·2	110	—	6·600	3·0
—	—	—	—	—	—	6·500	3·0
—	—	—	—	—	—	6·500	3·0; thyroid stopped.
—	—	—	—	—	—	—	
—	—	—	—	—	—	—	
—	—	—	—	—	—	6·350	
—	—	—	—	—	—	6·400	
—	—	—	—	—	—	6·450	
—	—	—	—	—	—	6·500	
—	—	—	—	—	—	—	Operation II; remnant of pancreas removed.
—	—	—	—	—	—	6·250	
—	—	—	—	—	—	—	
—	—	—	—	—	—	6·000	Very well, takes food readily.
—	—	—	—	—	—	—	
—	—	—	—	—	—	5·800	
—	—	—	—	—	—	—	Some pus discharge from scar of operation I.
—	—	—	—	—	—	5·650	Bright and active.
—	—	—	—	—	—	5·600	
—	—	—	—	—	—	—	Abdominal wound healed.
—	—	—	—	—	—		
—	—	—	—	—	—	5·350	Slight diarrhoea.
—	—	—	—	—	—	—	
—	—	—	—	—	—	5·200	Somewhat weak.
—	—	—	—	—	—	—	Died under anaesthetic. Only 4 hours urine was passed.

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XVIII. THE REACTION AND CALCIUM CONTENT OF MILK AS FACTORS IN THE COAGULATION PROCESS.

By THOMAS HUGH MILROY.

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(*Received April 12th, 1915.*)

The two main factors which govern the coagulant action of rennin on caseinogen are the reaction and the calcium content of the medium. Within recent years the influence of variations in the reaction upon this process have been studied especially by van Dam [1908], Allemann [1912] and Michaelis and Mendelssohn [1913], all of whom have employed the electrometric method for the estimation of the actual acidity or hydrogen ion concentration, [H˙][1]. Van Dam, who adopted a rather ingenious method of determining the coagulation time in specimens of milk which were only slightly diluted, came to the conclusion that the rate of coagulation was directly proportional to the [H˙] for the comparatively limited range which he investigated. After studying the action of $CaCl_2$ upon the coagulation period, he came to the conclusion that in so far as this salt raised the [H˙] of the mixture, it also hastened the formation of soluble casein from caseinogen. The specific action of the calcium was dependent not upon the calcium salts in solution in the milk, but upon the amount of calcium in colloidal combination. Allemann estimated the [H˙] of milk after the addition of equivalent amounts of hydrochloric and acetic acids and found that the [H˙] was the same for equivalent concentrations of the two acids although the degree of dissociation in the two cases was so different. This is evidently due to the fact that the milk acts as a reaction regulator from its alkaline phosphate content and the regulating power is lost when all the secondary is transformed into primary phosphate, acid precipitation of the caseinogen occurring at this point. The author also pointed out that prior to the precipitation of caseinogen by acid—stated quantitatively, with lower [H˙] than $1{\cdot}25 \,.\, 10^{-5}$ N

[1] [H˙] = Hydrogen ion concentration.

$p_{H^{\cdot}}$ = The negative exponent of the same when the normality is expressed as a power of 10.

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(from $0{\cdot}64 \,.\, 10^{-5}$ to $1{\cdot}25 \,.\, 10^{-5}$)—a zone of opalescence is met with and it is within this zone that the coagulant action of rennin takes place.

Michaelis and Mendelssohn investigated the subject in rather a different way from the others. They determined the optimum zones of [H$^{\cdot}$] for the coagulant action of rennin and the precipitation of caseinogen by acid. They found the optimum zone for rennin action in the presence of soluble calcium salts to lie between $0{\cdot}4 \,.\, 10^{-6}$ and $1 \,.\, 10^{-6}$ N. It was not possible to narrow this zone down further, but in their opinion $1 \,.\, 10^{-7}$ and $3 \,.\, 10^{-6}$ lay outside the region of optimal activity. The optimum zone for acid precipitation of caseinogen in pure solution or in milk was found to lie about $2{\cdot}5 \,.\, 10^{-5}$ N, while in $m/40$ $CaCl_2$ solution optimum precipitation was obtained with higher [H$^{\cdot}$], namely about $3 \,.\, 10^{-4}$ N. The authors therefore state that there is a region between 10^{-6} and 10^{-5} in which neither optimal rennin action nor optimal acid precipitation takes place. They therefore do not regard the zone of rennin action as simply an extension of the acid precipitation zone. Making use of acetate regulator mixtures to eliminate the rise in [H$^{\cdot}$] produced by the addition of $CaCl_2$, they came to the conclusion that the $Ca^{\cdot\cdot}$ action in rennin coagulation is not simply an expression of the [H$^{\cdot}$] rise. In studying the parts played by variations in [H$^{\cdot}$] and in the soluble calcium content on the coagulation process, it is essential to devise experimental conditions which render it possible to effect a change in the one without at the same time altering the other. It is necessary therefore to avoid the [H$^{\cdot}$] variations produced by the addition of calcium chloride to milk, and hence the use of regulator mixtures is required. In the investigation however of the rennin coagulation process, it is also necessary to determine whether during the course of enzyme action there is any change in [H$^{\cdot}$] such as occurs, for example, in the case of peptic digestion [Sörensen, 1908, 1912; Christiansen, 1912], in which at the outset the combining power of the digestion products for bases is increased and subsequently decreased. In the case of such an investigation, as also in the study of the effects produced on [H$^{\cdot}$] by substances which accelerate or retard coagulation, it is necessary to avoid the use of reaction regulators.

At the outset therefore the [H$^{\cdot}$] of fresh milk was determined before and during the course of digestion and without the addition of a reaction regulator mixture.

The method employed for the determination of the [H$^{\cdot}$] was the electrometric one as described by Sörensen and others. I have given certain details as to my own method of procedure in another paper [1914]. It is quite

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unnecessary to give a list of all the determinations of the [H˙] of fresh milk, but as the values depend partly upon the method employed for saturating the milk specimen in the electrode with hydrogen, it is necessary to refer briefly to the effect of bubbling the gas through the milk. When one employs the "stationary" hydrogen electrode (as used by Hasselbalch, Michaelis and others) the values are slightly higher than when the gas is bubbled through the milk, even when the precaution is taken of passing the gas through a series of wash bottles containing milk before it enters the electrode. The fall in [H˙] is evidently due to the removal of CO_2, but this can be greatly diminished by passing the gas through a number of milk tubes before it enters the electrode.

The [H˙] of Fresh Undiluted Milk.

From a very large number of specimens examined, the range of [H˙] in fresh milk was found to be from $0{\cdot}158 \,.\, 10^{-6}$ N (p_H. 6·80) to $0{\cdot}310 \,.\, 10^{-6}$ N (p_H. 6·50) as determined by the stationary hydrogen electrode method. When hydrogen is bubbled through the milk in the electrode, with the precautions taken which have just been mentioned, the [H˙] range is from $0{\cdot}140 \,.\, 10^{-6}$ to $0{\cdot}158 \,.\, 10^{-6}$ N. The great majority of the fresh milk specimens have a [H˙] from $0{\cdot}18 \,.\, 10^{-6}$ (p_H. 6·75) to $0{\cdot}25 \,.\, 10^{-6}$ (p_H. 6·60) as determined by the stationary electrode method.

The [H˙] during the Coagulation Process.

This is most easily estimated either in milk which has been diluted or in milk which has been first rendered non-coagulable by the addition of alkaline oxalate. The coagulation process can be readily followed in the oxalated milk on the addition of $CaCl_2$. The addition of potassium oxalate to milk causes a fall in [H˙] owing to the hydrolytic dissociation of the salt.

Thus 1 cc. $K_2C_2O_4 \,.\, 4H_2O$ (3 %) added to 10 cc. milk—i.e. about $m/80$ oxalate concentration—gives rise to a fall in [H˙] from $0{\cdot}19 \,.\, 10^{-6}$ N to $0{\cdot}06 \,.\, 10^{-6}$ (p_H. 6·70 to 7·22). That is to say the addition of a sufficient quantity of potassium oxalate to render milk non-coagulable lowers the [H˙] to such an extent that the change in reaction alone, apart altogether from the calcium precipitation, would place the milk outside the range of Michaelis' coagulation zone. One must therefore bear in mind that the depression of coagulability produced by the addition of oxalate must be in part due to the lowered [H˙].

If rennin be added to an "oxalate" milk and the mixture kept in a water-bath at 38° the [H˙] is not found to change during the period of digestion.

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An example may be given of such a milk specimen which remained unclotted throughout the experiment.

TABLE I.

	$[H^{\cdot}] \times 10^6$ N.	$p_{H^{\cdot}}$
Fresh milk	0·477	6·70
	$[H^{\cdot}] \times 10^7$	$p_{H^{\cdot}}$
Same containing $m/80$ $K_2C_2O_4$, after 10 min. digestion	0·707	7·15
" " " 20 "	0·724	7·14
" " " 30 "	0·724	7·14
" " " 60 "	0·741	7·13

When $CaCl_2$ is added to an "oxalate" milk the $[H^{\cdot}]$ rises, but the milk remains uncoagulable until the $[H^{\cdot}]$ reaches a higher level than $p_{H^{\cdot}}$ 6·86. The following examples of different specimens of milk are given in order to show the effects produced by oxalate and $CaCl_2$ on the $[H^{\cdot}]$ of milk subjected throughout to the action of rennin at 38°.

The E.M.F. of the fluid is given at different periods and the calculated $p_{H^{\cdot}}$ in order that any change in the $[H^{\cdot}]$ throughout the course of the experiment may be observed.

TABLE II.

(1) Milk 10 cc., $K_2C_2O_4$ (3 %) 1 cc., Rennin (0·25 %) 1 cc. at 38°.

Period of digestion in minutes	E.M.F.	$p_{H^{\cdot}}$	Remarks
5	0·7552	7·23	fluid
10	0·7546	7·22	"
15	0·7544	7·22	"
	added 1 cc. $CaCl_2$ (1 %)		
	0·6962	6·21	curdling
6	0·6948	6·18	clotted
10	0·6947	6·18	"
20	0·6949	6·18	"

(2) Another specimen of milk containing the same amount of oxalate and rennin at 38°.

Period of digestion in minutes	E.M.F.	$p_{H^{\cdot}}$	Remarks
1	0·7508	7·15	fluid
5	0·7500	7·14	"
10	0·7496	7·13	"
15	0·7494	7·13	"
	added 0·75 cc. $CaCl_2$ (1 %)		
1	0·7103	6·46	fluid
2	0·7110	6·47	"
5	0·7127	6·49	curdling
10	0·7135	6·50	clotted
15	0·7135	6·50	"
30	0·7135	6·50	"

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(3) Another specimen of oxalate milk and rennin at 38°.

Period of digestion in minutes	E.M.F.	p_H.	Remarks
1	0·7420	7·00	fluid
5	0·7400	6·97	,,
10	0·7391	6·97	,,
	added 0·3 cc. $CaCl_2$		
1	0·7343	6·87	fluid
3	0·7340	6·86	,,
6	0·7335	6·86	,,
10	0·7338	6·86	,,
	again added 0·3 cc. $CaCl_2$		
10	0·7166	6·56	clotted
20	0·7141	6·52	,,
25	0·7140	6·52	,,

Other specimens gave similar results. It is evident in the first place that the oxalate lowers the [H˙] while the $CaCl_2$ raises it and that coagulation occurs when the [H˙] reaches the level of that possessed by a normal coagulable milk.

During the period, however, when the ferment is acting on milk either containing an excess of oxalate or of $CaCl_2$ there is no definite change of reaction.

It is certain that in specimen (3) after the addition of 0·3 cc. $CaCl_2$, when the [H˙] has been raised approximately to the lowest level of the optimum coagulation zone, viz. p_H. 6·86–6·87, ferment action must have been proceeding during the ten minutes in which the potential estimations were made and yet there was no appreciable alteration in the E.M.F.

Therefore during the early period of the rennin action prior to the separation of a clot there is no indication of accompanying [H˙] alterations such as are evident in pepsin action. Again, in the actual passage from the fluid to the clotted state there is also no definite indication of an alteration in the [H˙] such as might occur from the fixation of calcium by the soluble casein giving rise to an increase in the [H˙] of the fluid.

Effect of Variations in the Calcium Content and the Reaction of Milk on Coagulability.

As alkaline oxalate in addition to its effect upon the soluble calcium content of the milk also lowers the [H˙], it was necessary either to counteract this effect upon the [H˙] as, for example, by the addition of oxalic acid, or to adopt some other method of diminishing the soluble calcium content. It has been for a long time well known that milk which has been kept at a

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temperature over 80° C. is gradually rendered less and less coagulable by rennin. Otto de Vries and Boekhout [1901] stated that the calcium content of such poorly coagulating milk is not lower than that of normal milk, although they granted that the addition of $CaCl_2$ rendered the milk coagulable. This was not due, however, in their opinion to an increased soluble calcium content of the milk, and they therefore concluded that the rise in acidity was the governing factor. Van Dam estimated the total calcium content of milk of normal coagulability and that of milk which either did not coagulate at all or only after a longer period than normal, and he found that the former contained about 0·154–0·174 % CaO, while the latter contained 0·135–0·150 % CaO. Klein and Kirsten [1901] state that heated milk on addition of $CaCl_2$ clots more readily with rennin than unheated milk.

Milk rendered uncoagulable by heating.

The milk was kept at a temperature slightly below boiling point for one hour, by placing it in the inner vessel of a double saucepan, the outer one containing boiling water. Such milk, which shows greatly reduced coagulability, was made use of in the experiments which follow. After the milk had been heated for one hour it was cooled, made up to the original volume and filtered after again being warmed. The first point to determine was the [H˙] of the heated milk as compared with that of the same milk prior to heating. So far as I know, the [H˙] of heated milk has not hitherto been determined.

The following table gives the [H˙] of different specimens of milk before and after heating.

TABLE III.

[H˙] *of Milk before and after heating.*

	Before			After		
	E.M.F.	$[H^{\cdot}] \times 10^7$ N	$p_{H^{\cdot}}$	E.M.F.	$[H^{\cdot}] \times 10^7$ N	$p_{H^{\cdot}}$
(1)	0·7174	2·63	6·58	0·7126	3·25	6·49
(2)	0·7254	1·90	6·72	0·7162	2·81	6·55
(3)	0·7274	1·77	6·75	0·7174	2·63	6·58
(4)	0·7273	1·77	6·75	0·7190	2·51	6·60
(5)	0·7304	1·58	6·80	0·7190	2·51	6·60
(6)	0·7310	1·55	6·81	0·7250	1·95	6·71

In every case the heated milk shows a higher [H˙] than the same milk before heating.

The [H˙] of heated milk specimens remains also much more constant than that of fresh milk. Heating therefore produces exactly the opposite effect on the [H˙] from that produced by the addition of potassium oxalate.

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In order to understand the cause of this rise in acidity attention requires to be directed to the calcium content of the milk. The percentage of CaO in different specimens of milk before and after heating is shown in the following table.

TABLE IV.

Percentage of CaO *in Milk before and after heating.*

	Before CaO %	After CaO %
(1)	0·1738	0·1345
(2)	0·1738	0·1524
(3)	0·1630	0·1416
(4)	0·1577	0·1437
(5)	0·1542	0·1447

These results show an average of 0·1645 % CaO in unheated and 0·1434 % CaO in heated milk. The rise in [H˙] which occurs on heating milk is evidently due therefore to the separation of calcium phosphate in more basic form than it previously existed in the milk, the transformation probably being of the following nature:

$$3CaHPO_4 + Na_2HPO_4 = Ca_3(PO_4)_2 + 2NaH_2PO_4.$$

The removal of carbonic acid from the milk by heating lowers the solubility of the calcium phosphates.

According to Conradi [1901] the diminished coagulability of heated milk with rennin goes hand in hand with an increase in coagulability on heating with calcium chloride. This is undoubtedly the case, for if one takes two 5 cc. specimens of fresh and heated milk, one finds that after adding 0·5 to 0·6 cc. $CaCl_2$ (1 %) to each, the heated specimen coagulates on boiling, while the fresh one does not coagulate until 0·8–0·9 cc. is added.

Action of rennin on heated milk.

As above stated, the coagulant action of the ferment on heated milk is very much diminished and that this decrease in activity is not due to the alteration in reaction is evident from the fact that the increase in [H˙] produced by heating milk ought of itself to increase the coagulability. That the diminished coagulability is due to the lowered calcium content of the milk is evident from the fact that on raising the percentage of calcium in heated milk to the same level as in the milk before heating, the milk coagulates as readily as the fresh milk. As already stated, however, the addition of calcium chloride to the milk raises the [H˙] unless the milk be diluted with some reaction regulator such as the acetate mixture employed by Michaelis.

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When heated milk is diluted with sodium acetate ($m/4$) the [H˙] is lowered owing to the hydrolytic dissociation of the alkaline acetate. On adding $CaCl_2$ to this mixture the [H˙] at first rises owing to the lower dissociation constant of the calcium acetate; with further additions of $CaCl_2$, however, the [H˙] only alters slightly. Thus, for example, the $p_{H^{\cdot}}$ values of certain acetate and acetate-$CaCl_2$ milk mixtures are as follows:

		$p_{H^{\cdot}}$
(1)	Heated milk (not diluted)	6·56
(2)	Heated in $m/4$ sodium acetate (milk 1 in 4 dilution)	6·97
(3)	Same as (2) but containing $m/200$ $CaCl_2$	6·81
(4)	Same as (2) but containing $m/40$ $CaCl_2$	6·73

In the subsequent experiments N sodium acetate was added to the milk.

Addition of $CaCl_2$ *to heated milk.*

The following series illustrates the action of $CaCl_2$ on the coagulability of heated milk diluted as follows,

Milk	10 cc.
Sodium acetate N ..	2 cc.
Water to	40 cc.

To this mixture various amounts of $CaCl_2$ were added and the [H˙] and coagulation time noted.

TABLE V.

1 % $CaCl_2$	E.M.F.	[H˙] × 10^6 N	$p_{H^{\cdot}}$	Coagulation time in minutes
2 cc.	0·7307	0·15	6·81	6
4	0·7257	0·19	6·72	5
6	0·7207	0·23	6·63	3
8	0·7198	0·24	6·62	2·5
10	0·7195	0·24	6·61	2

Although it is evident that the addition of the $CaCl_2$ solution has produced a rise in [H˙], this is inappreciable after the initial increase. The shortening in the coagulation period to one-third of the original has taken place with an 80 % increase in the [H˙]. In many cases the rise in [H˙] produced by the addition of $CaCl_2$ is even less marked, as in the specimens previously referred to where a $m/200$ $CaCl_2$ in milk-sodium acetate had a $p_{H^{\cdot}}$ of 6·81, while a $m/40$ $CaCl_2$ showed a rise in [H˙] only to $p_{H^{\cdot}}$ 6·73. Yet in this case the shortening in the coagulation time was approximately the same as in the last mentioned series.

The factor concerned in the shortening of the coagulation period in this series has evidently not been the slight rise in [H˙] because such a rise

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otherwise produced than by addition of $CaCl_2$ would scarcely have shortened the coagulation period.

If we now determine the rise in [H˙] which is required in order to render heated milk coagulable without the further addition of $CaCl_2$ we obtain results which make it evident that the calcium chloride does not produce its effect simply from the rise in [H˙] which it produces.

The coagulation zone of heated milk diluted with sodium acetate and acetic acid.

Heated milk may be again rendered coagulable by rennin not only by an increase in the soluble calcium content without an appreciable rise in the [H˙], but also by a rise in the [H˙] without an increase in the soluble calcium content except in so far as the increasing acidity may produce a slight effect.

When heated milk is diluted with sodium acetate, the [H˙] falls. This fall is prevented by the addition of acetic acid. The following table gives the [H˙] and observations on the coagulation of milk-acetate-acetic acid mixtures of different [H˙].

TABLE VI.

Milk 10 cc., Sodium acetate (N) 10 cc., Rennin (0·25 %) 2 cc., Water up to 40 cc.

	E.M.F.	$[H^{\cdot}] \times 10^7$ N	p_H.	
Without acid	0·7416	1	7·00	no clot
0·6 cc. acetic acid (N)	0·6920	7·24	6·14	,,
1·2 ,, ,,	0·6717	16·69	5·78	clotted
1·5 ,, ,,	0·6661	20·42	5·69	,,
2 ,, ,,	0·6577	28·84	5·54	,,

From a comparison of Tables V and VI it is evident that coagulation takes place at a much lower [H˙] when $CaCl_2$ is added to the heated milk than when acetic acid is added, namely in the case of the former with [H˙] from $0{\cdot}15$ to $0{\cdot}24 \times 10^{-6}$ N, in the latter on the other hand only when the [H˙] is higher than $0{\cdot}724 \times 10^{-6}$. There are therefore two variables to be considered in the case of the rennin action on caseinogen, namely the [H˙] of the fluid and its calcium content.

Before discussing the relationship of these variables to one another, it is advisable to refer briefly to the acid precipitation zone of caseinogen in the absence of ferment, in order to see whether the ferment zone is not after all simply an extension of the acid precipitation zone.

If heated milk be diluted with sodium acetate-acetic acid mixture as in the preceding series and the [H˙] determined at the point when caseinogen

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is precipitated from the milk, one obtains results which show that the precipitation zone lies on the acid side of the coagulation zone. The milk-acetate-acetic acid mixtures were kept at 38°.

Milk	10 cc.
Sodium acetate (N)	10 cc.
Acetic acid N from	1 cc. upwards
Water to	40 cc. at 38°

Until the [H˙] reached $0{\cdot}95 \,.\, 10^{-5}$ there was no sign of a precipitate. The figures below give the acidities at which precipitation takes place.

E.M.F.	$[H^{\cdot}] \times 10^5$ N	p_H.	Remarks
0·6377	0·630	5·20	No ppt. in 1 hour
0·6275	0·955	5·02	ppt. in 6 min.
0·6236	1·122	4·95	ppt. in 4 min.

On repeating this experiment but with 2 cc. $CaCl_2$ (1 %) added the acid precipitation of caseinogen took place at practically the same [H˙] as in the absence of the $CaCl_2$. A precipitate was obtained in 6 min. with a [H˙] of $1{\cdot}04 \,.\, 10^{-5}$ (p_H. 4·98).

Michaelis and his fellow workers give, as the most favourable [H˙] for the precipitation of caseinogen in the absence of $CaCl_2$, $2{\cdot}5 \,.\, 10^{-5}$ N (p_H. 4·60), which is a more acid reaction than the one I have just given. They employed, however, caseinogen solutions, and the conditions as regards temperature of mixture and sodium acetate concentration were different in the two cases.

Michaelis and Mendelssohn found that in the presence of $CaCl_2$ (in greater concentrations than those used by me) the acid precipitation zone of caseinogen is shifted still more to the acid side.

Although there is no doubt that the acid precipitation zone of heated milk lies some distance on the acid side of the coagulation zone, it is certain that with still further lowering of the calcium content of the milk, the zone of rennin action would approach more nearly that of acid precipitation.

The [H˙] of milk therefore requires to be highest for the precipitation of caseinogen by acid from solutions of its salts.

The separation of caseinogen from its solutions by the action of rennin takes place at lower [H˙], the exact point at which coagulation takes place being dependent upon the soluble calcium content. It is highest when the calcium content is low and gradually falls as the latter is raised.

What then are the exact relations of these two variables to one another?

The value of the one variable [H˙], which corresponds to a definite value of the other variable (calcium) for a fixed coagulation time, has been determined in a series of milk-acetate-acetic acid mixtures. The coagulation time

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which was taken was 5 min., and the calcium content and [H˙] of the various milk-acetate mixtures determined which showed coagulation by rennin within that time.

Beginning with a milk-acetate mixture made up as follows:

Heated milk	10 cc.
Sodium acetate (N)	10 cc.
Rennin (0·25 %)	2 cc.
Water to	40 cc.

the [H˙] and CaO percentage of the diluted mixture were determined and found to be $p_{H^{\cdot}}$ 7·00 and 0·035 % CaO respectively. This mixture did not clot.

To a series of milk-acetate mixtures of the above composition acetic acid (normal) was added (starting with 0·1 cc.) until one was obtained which clotted in five minutes.

It was found that a milk-acetate-acetic mixture of [H˙] $0{\cdot}112 \,.\, 10^{-5}$ clotted in ten minutes; one of $0{\cdot}157 \,.\, 10^{-5}$ in seven minutes; and one of $0{\cdot}177 \,.\, 10^{-5}$ in five minutes.

Thus a milk specimen of 0·035 % CaO required to have the [H˙] raised to $0{\cdot}177 \,.\, 10^{-5}$ to obtain coagulation in five minutes.

The calcium content of the diluted milk was then gradually increased by the addition of small quantities of $CaCl_2$ (1 %). After each addition of $CaCl_2$ a series of milk-acetate-acetic acid mixtures of the same calcium content but of different [H˙] was prepared, and the [H˙] of the one which showed coagulation in five minutes was accurately determined.

The following table gives the results:

	CaO %	$[H^{\cdot}] \times 10^6$ N	$p_{H^{\cdot}}$
(1)	0·035	1·778	5·75
(2)	0·036	1·660	5·78
(3)	0·038	0·871	6·06
(4)	0·042	0·309	6·51
(5)	0·050	0·230	6·64
(6)	0·060	0·186	6·73
(7)	0·073	0·154	6·81
(8)	0·085	0·135	6·87
(9)	0·122	0·126	6·90
(10)	0·135	0·107	6·97

From this table it is evident that when the calcium content is brought below 0·06 % and especially when it has fallen to 0·042 % the [H˙] requires to be proportionately much more increased in order to enable coagulation to take place within the five-minute period. Thus in the range from 0·060 to 0·035 % CaO, the [H˙] requires to be increased tenfold. This range in

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calcium content represents the difference between fresh milk and heated milk diluted to the same extent with the acetate mixture.

The specimens of heated milk diluted with the acetate mixtures which possess hydrogen ion concentrations corresponding to those of fresh coagulable milk have a CaO percentage similar to those of fresh coagulable milk diluted to the same extent, namely specimens (4), (5) and (6). In the specimens above (6) NaOH (N/10) was added in order to obtain the lower $[H^{\cdot}]$.

The Rôle of the Calcium Salts in the Formation of Soluble Casein.

In the preceding experiments attention has been merely directed to the separation of the casein clot, and practically no reference was directly made to the earlier part in the coagulation process, namely the formation of soluble casein. The part which the calcium content of the milk plays in this earlier process is difficult to dissociate from that played by the accompanying reaction changes.

The question to decide is whether the transformation of caseinogen to soluble casein takes place at the same rate in the case of a milk specimen of lowered calcium content as in one possessing a normal amount. Various methods were employed in order to decide this question. A few examples illustrating these methods may be given.

Two diluted milk specimens of the following composition were taken:

(1)	Milk (heated)	10 cc.	(2) Same but without $CaCl_2$
	$CaCl_2$ (1 %)	2 cc.	
	Rennin (0·25 %)	2 cc.	
	Water to	40 cc.	

These specimens were kept at 38°. Clotting took place in specimen (1) in two minutes. Immediately on the appearance of the clot in this specimen, 2 cc. $CaCl_2$ (1 %) were added to the other specimen, which up to this time had shown no clot. Coagulation occurred two minutes later. On this being repeated with a smaller amount of rennin solution, coagulation occurred in specimen (1) in 3·5 minutes, and in specimen (2) 3·5 minutes after the addition of $CaCl_2$. That is to say in neither of these cases did the preliminary digestion in a diluted milk specimen without the addition of $CaCl_2$ shorten the subsequent period of coagulation after the addition of $CaCl_2$.

One must bear in mind, however, that the addition of $CaCl_2$ not only increases the calcium content of the milk but it also, especially in milk simply diluted with water, raises the $[H^{\cdot}]$, and thus specimen (1) was

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placed under more favourable circumstances, as regards reaction, for the activity of the rennin. The addition of water alone lowers the [H·] and so specimen (2) was placed under more unsatisfactory conditions for the enzyme action.

It is necessary therefore to compare two diluted milk (heated) specimens of approximately the same [H·], the one containing $CaCl_2$, the other without this addition as was done in the following experiment:

(1)	Milk (heated)	10 cc.	(2) Same but without $CaCl_2$
	Sodium acetate (N)	10 cc.	
	Acetic acid (N/10)	0·5 cc.	
	$CaCl_2$ (1 %)	1 cc.	
	Rennin (0·25 %)	2 cc.	
	Water to	40 cc.	

Both specimens had a [H·] of approximately $2 \cdot 10^{-7}$ N. Specimen (1) clotted in 5·75 min. Immediately on appearance of coagulation in this specimen 1 cc. $CaCl_2$ (1 %) was added to the other, which at this time showed no sign of coagulation. Coagulation occurred in this second specimen 2·75 min. later. That is to say the preliminary digestion with rennin in a milk-acetate mixture to which no $CaCl_2$ was added has shortened the period of coagulation after the addition of $CaCl_2$ by rather more than one half. Evidently therefore, although as was to be expected the transformation of caseinogen into soluble casein takes place in such a mixture when no $CaCl_2$ has been added, the rate of transformation is slower than in the presence of a small quantity of that salt, even when the [H·] in both cases is the same.

That the addition of $CaCl_2$ does not act simply as a precipitant of soluble casein is shown also from the following experiment.

20 cc. heated milk were taken, diluted 1 in 4 with water and kept at 38°. The mixture was divided into three portions. One of these was poured into a flask containing 2 cc. rennin and 1 cc. $CaCl_2$ (1 %), the other two portions into flasks containing 2 cc. rennin alone, and all were kept at 38°. The first specimen clotted in four minutes. One of the remaining specimens was then brought to boiling point, in order to destroy the rennin, and then cooled to 38°, while the third specimen was kept at 38°. When 1 cc. $CaCl_2$ (1 %) was added to each of these specimens after four minutes, the one in which the rennin had been destroyed did not clot, while the third specimen clotted in rather more than one minute.

In both of these specimens during the four minutes' action of rennin the caseinogen must have been acted upon by the ferment to such an extent that on the addition of 1 cc. $CaCl_2$ coagulation would take place after the

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lapse of little over a minute, but this occurred only in the case where the ferment was allowed to continue its action during that extra period.

It is evident therefore that the action of the soluble calcium salt is not simply to complete a process the earlier stages of which do not require its presence.

From the beginning of the ferment action until its close the rate of transformation of caseinogen into the final product of digestion is dependent upon the calcium content and reaction of the medium.

Conclusions.

1. During the course of rennin action there is no change in the [H˙] of milk, either in the earlier stage or in the actual separation of the clot.

2. The addition of an alkaline oxalate to milk lowers, while that of $CaCl_2$ raises, the [H˙].

3. Fresh milk which has been subjected to a temperature slightly below boiling point for one hour shows a rise in [H˙], and a fall in the calcium content. Such milk is only very slowly acted upon by rennin.

4. The coagulability of heated milk may be raised either by the addition of $CaCl_2$ or by raising the [H˙]. The former does not act simply by raising the [H˙] nor the latter from its effect upon the soluble calcium content.

5. The acid precipitation zone of caseinogen lies on the acid side of the rennin zone of action, but the latter gradually approaches the former as the calcium content of the mixture is lowered, so that in all probability the latter is an extension of the former towards the neutral point.

6. Calcium chloride, apart from its effect upon [H˙], increases the activity of the rennin ferment from the beginning of the digestion process.

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XIX. THE RELATIONSHIP OF CREATINURIA TO CHANGES IN THE SUGAR CONTENT OF THE BLOOD.

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Previous Work and Object of present Research.

An increase in the normal sugar content of the blood has long been known to be an associated phenomenon of certain pathological conditions, especially pancreatic diabetes or the glycosuria artificially produced by the injection of certain drugs, e.g. uranium. Of recent years it has been shown that a diminution in the normal sugar content, or a hypoglycaemia, may follow the injection of certain other drugs, which cause considerable hepatic disturbance accompanied by the excretion of creatine. Some previous work by the writer [MacAdam, 1913] in corroboration of the hypothesis put forward by Cathcart [1909] that carbohydrates are intimately associated with the metabolism of creatine may explain the onset of this creatinuria.

Frank and Isaac [1911] by the administration of phosphorus to rabbits produced a distinct hypoglycaemia along with marked liver necrosis. The injection of hydrazine sulphate, which has a specific action in causing hepatic necrosis [Wells, 1908], leads to changes in the percentage of sugar in the blood, as shown by Underhill [1911; Underhill and Fine, 1911] both in dogs and rabbits. As the result of numerous experiments on dogs he found that a dose of 50 mgrms. per kilo. invariably produced an appreciable hypoglycaemia, although considerable variations in the degree of diminution of the sugar content occurred. His experiments with rabbits were not so conclusive. On the administration of hydrazine in doses of 50 mgrms. per kilo. a constant decrease in the sugar content was not obtained; for, although a hypoglycaemia of varying amount was found to occur in the

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majority of the experiments, in others the blood sugar remained unaltered. On the administration of 100 mgrms. per kilo. the animals died within 24 hours, and Underhill concludes "From this diversity of results it is obvious that the rabbit cannot be relied on invariably to exhibit hypoglycaemia after hydrazine injection."

In these experiments the relationship of hypoglycaemia to creatinuria was not considered, and no attempt was made by the administration of intermediate doses to allow for the idiosyncrasy of individual rabbits. The object of the following experiments has been to try to determine experimentally whether this irregular occurrence of hypoglycaemia in rabbits depends upon the dose of hydrazine injected, and if it is in any way associated with the simultaneous occurrence of creatine in the urine.

Experiments and Methods of Analysis.

The rabbit was kept in a small cage over a funnel leading to a vessel in which the urine was collected. 5 cc. of 5 % H_2SO_4 were placed in this vessel previous to collection to prevent alkaline changes in the urine. At first a difficulty was experienced in obtaining the urine creatine-free during the control days. By means of a carbohydrate-rich diet this was in most instances surmounted or at least so diminished that the amount of creatine still present was negligible. In a few cases persistent creatinuria was found to be dependent upon pregnancy, and the rabbits were accordingly discarded. No attempt was made to obtain the quantitative amounts of creatine and creatinine excreted, partly on account of unequal quantities of urine being passed during each 24 hours, partly because such absolute figures have no direct bearing on the object of the present research. *Percentage* results are dealt with in Table I, the relationship of the excretion of creatine to total creatinine being thereby shown.

Control estimations of the sugar content were first made from 7–10 days previous to the injection of a solution of hydrazine sulphate (2·5 %). This was done without doing any injury to the vascular system of the animal, for after some practice no difficulty was experienced in obtaining from 20–30 cc. of blood from the auricular vein. The blood was dropped directly into a 50 cc. flask containing 5 cc. of a 2 % solution of potassium oxalate, and by the addition of the requisite amount of distilled water from a burette to bring the total amount of fluid up to 50 cc., the quantity of blood obtained was accurately determined. In two of the experiments recorded where the rabbits after the administration of hydrazine became comatose, it was found

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impossible to obtain blood from the auricular vein, and hence it was drawn directly from the jugular vein and heart. The sugar content was estimated by the method of Allihn in terms of CuO.

The accuracy of the technique was tested in the case of a dog used for the purpose of another research [MacAdam, 1913] which became comatose within 24 hours after the injection of 90 mgrms. per kilo. of hydrazine. The dog was killed and from its jugular vein two quantities of blood, 32·2 cc. and 60 cc., were obtained, the sugar content of which was found to be 0·064 % and 0·068 % respectively. The sugar content of the normal dog is constant within narrow limits and averages 0·1 %, so that it is interesting to note that the above estimations corroborate the work of Underhill [1911], who found a sugar content of 0·05 % in the blood of a dog one day after the injection of 100 mgrms. hydrazine per kilo. of body weight.

In the initial experiments, doses of 50 mgrms. per kilo. body weight were administered, and 48 hours later blood was withdrawn, as above mentioned, for sugar estimation, while daily estimations of the urinary excretion of preformed creatinine and creatine were carried out.

If no symptoms were exhibited, and no creatinuria resulted, the same rabbit was injected again but with a larger dose in order to try just to exceed the tolerance of the animal's tissues to the poison. The varying doses used in each experiment are recorded in the protocols.

Since the animals, which showed metabolic disturbance, refused all food after the injection, two experiments (X and XI) with the rabbits fasting for two days were carried out in order to eliminate the factor of starvation from the results.

TABLE I.

(*The days of the control period in each experiment during which an attempt was being made to obtain the urine creatine-free are indicated by Arabic numerals, while the Roman figures refer to the days following the injection of the hydrazine.*)

I. *Rabbit A.* Weight 3·20 kilos.

Day	Total Creatinine	Preformed C.	Creatine as Creatinine	% Creatine of Total Creatinine	% Sugar Content of blood	Remarks
	mgrms. in 100 cc. urine					
1	127·4	127·4	—	—	0·114	
2	101·5	98·3	3·2	3·1	—	
	Injection of 50 mgrms. Hydrazine sulph. per kilo.					Animal showed no metabolic disturbance.
II	87·0	87·0	—	—	0·108	

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TABLE I (*continued*)

II. *Rabbit B.* Weight 2·73 kilos.

Day	Total Creatinine	Preformed C.	Creatine as Creatinine	% Creatine of Total Creatinine	% Sugar Content of blood	Remarks
	mgrms. in 100 cc. urine					
1	71·0	65·5	5·5	7·4	0·095	
2	107·7	90·0	17·7	16·4	—	
3	—	—	—	—	—	
4	81·5	81·5	—	0·0	—	
	Injection of 50 mgrms. Hydrazine sulph. per kilo.					Nothing abnormal was detected in the rabbit's condition after injection, food being taken as usual.
I	72·5	74·1	—	—	—	
II	124·5	121·5	3·0	2·4	0·102	
III	110	115·0	—	—	—	

III. *Rabbit B.* Weight 2·47 kilos.

1	126·5	126·5	—	—	0·087	
2	107·5	98·5	9·0	8·3	—	
3	94·3	94·3	—	0·0	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
I	139·5	146·0	—	—	—	No metabolic disturbance noted.
II	124·5	126·5	—	—	0·107	
III	85·7	82·6	3·1	3·6	—	

IV. *Rabbit C.* Weight 1·77 kilos.

1	206·5	204·3	2·2	1·0	0·101	
2	87·3	87·3	—	—	—	
3	132·1	134·8	—	—	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
I	142·1[1]	135·0	7·1	5·0	—	Rabbit died within 12 hours of injection—no sugar estimation.

[1] Urine drawn from bladder post-mortem.

V. *Rabbit D.* Weight 2·32 kilos.

1	165·0	139·0	26·0	15·7	0·098	
2	—	—	—	—	—	
3	158·5	158·5	—	—	—	
4	72·0	73·5	—	—	—	
5	107·5	107·5	—	—	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
II	158·5	62·8	95·7	60·3	0·047	Rabbit showed considerable malaise, refusing all food after injection. Blood could not be obtained from auricular vein, and was taken from the jugular vein.

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TABLE I (*continued*)

VI. *Rabbit A.* Weight 2·82 kilos.

Day	Total Creatinine	Preformed C.	Creatine as Creatinine	% Creatine of Total Creatinine	% Sugar Content of blood	Remarks
	mgrms. in 100 cc. urine					
1	155·7	155·7	—	—	0·098	
2	128·4	125·2	3·2	2·4	—	
3	99·8	101·2	—	—	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
II	112·5	112·5	—	—	0·110	
III	154·0	156·5	—	—	—	No effects produced.

VII. *Rabbit A.* Weight 2·96 kilos.

1	171·0	162·0	9·0	5·2	—	
2	—	—	—	—	—	
3	62·5	64·5	—	—	—	
4	149·0	144·0	5·0	3·3	—	
	Injection of 100 mgrms. Hydrazine sulph. per kilo.					
II	217·0	135·0	82·0	37·8	0·150	The rabbit from the first day after injection showed considerable malaise. Blood was obtained with difficulty from the auricular vein, only 19·7 cc. being obtained.
III	279·0	208·0	71·0	25·4	—	

VIII. *Rabbit B.* Weight 2·87 kilos.

1	159·6	148·7	10·9	6·8	—	
2	—	—	—	—	—	
3	78·3	78·3	—	—	—	
4	122·1	122·1	—	—	—	
	Injection of 90 mgrms. Hydrazine sulph. per kilo.					
II	172·9	114·2	58·7	33·9	0·060	The usual symptoms of metabolic disturbance. Rabbit recovered, but was not used for further experiment.
III	96·3	68·4	27·9	20·9	—	

IX. *Rabbit F.* Weight 1·70 kilos.

1	137·3	137·3	—	—	0·09	
2	106·0	103·8	2·2	2·0	—	
3	143·4	145·2	—	0·0	—	
	Injection of 75 mgrms. Hydrazine sulph. per kilo.					
I	43·6	39·1	4·5	10·3	0·094	Rabbit found comatose 24 hours after injection: 45 cc. of urine were removed from the bladder—acid in reaction. The blood was obtained directly from the heart.

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TABLE I (*continued*)

X. *Rabbit G.* Weight 2·14 kilos.

Day	Total Creatinine	Preformed C.	Creatine as Creatinine	% Creatine of Total Creatinine	% Sugar Content of blood	Remarks
	mgrms. in 100 cc. urine					
1	66·5	63·5	3·0	4·5	0·104	
2	130·0	130·0	—	—	—	
3	—	—	—	—	—	
4	106·5	102·6	3·9	3·6	—	
I	Fasting experiment					
II	187·4	143·5	43·9	23·4	0·140	After 2 days' starvation.
III	124·2	101·6	22·6	18·2	—	

XI. *Rabbit K.* Weight 1·91 kilos.

1	126·0	128·2	—	—	0·092	
2	137·0	137·0	—	—	—	
I	Fasting experiment					
II	132·5	95·7	36·8	27·7	0·116	After 2 days' starvation.
III	87·2	59·6	27·6	31·6	—	

XII. *Rabbit G.* Weight 2·0 kilos.

1	124·5	115·5	9·0	7·2	0·095	
2	97·4	99·2	—	—	—	
3	131·4	128·0	3·4	2·5	—	
Injection of 75 mgrms. Hydrazine sulph. per kilo.						
II	92·8	58·8	34·0	36·6	—	Rabbit showed usual symptoms of considerable malaise.
III	128·5	95·0	33·5	26·1	0·071	

XIII. *Rabbit M.* Weight 2·54 kilos.

4	102·5	74·3	28·2	27·5	0·105	
5	144·5	135·0	9·5	6·5	—	
6	58·6	54·3	4·3	7·3	—	
Injection of 50 mgrms. Hydrazine sulph. per kilo.						
II	108·0	84·5	23·5	21·7	0·114	Animal aborted after withdrawal of blood.

XIV. *Rabbit K.* Weight 1·7 kilos.

1	—	—	—	—	0·101	
2	134·0	136·0	—	—	—	
3	98·4	98·4	—	—	—	
Injection of 90 mgrms. Hydrazine sulph. per kilo.						
II	89·4	56·4	33·0	37·0	0·042	Rabbit was found comatose on morning of 2nd day, and 18·3 cc. of blood removed from the jugular vein. Urine was obtained directly from the bladder—acid in reaction

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Summary of Results.

In the four experiments (I, II, III, VI) where the hydrazine administered was insufficient to produce any obvious metabolic disturbance no creatine was excreted, while the sugar content of the blood, as compared with the control estimation previously made, was within normal limits, when due allowance has been made for experimental error.

Of the six experiments, where creatinuria followed hydrazine injection, four (V, VIII, XII, XIV) showed a condition of hypoglycaemia; one (VII) a state of hyperglycaemia, while in the remaining one (IX) the sugar content remained unchanged. In this last experiment it may be noted that the sugar estimation was carried out on blood obtained directly from the heart 24 hours after the administration of the hydrazine; while the percentage $\frac{\text{creatine}}{\text{total creatinine}}$ was small, viz. 10%, when compared with the figures obtained in the "hypoglycaemia" experiments. Indeed it was not much higher than the percentage occasionally recorded during the control days.

In Experiment XIII, where the rabbit aborted after the withdrawal of the second quantity of blood, and where pregnancy had not been previously noticed, there is little difference between the two estimations although there is a distinct increase in the creatine percentage of the total creatinine.

In the two fasting experiments, the rabbit of Experiment X showed quite a marked hyperglycaemia, while in XI the sugar content of the blood showed a slight increase when compared with the control estimation. In both a marked creatinuria resulted.

In the remaining experiment (IV) Rabbit C died within 12 hours. No blood was obtained, while the urine drawn from the bladder post-mortem showed a trace of creatine. This was the only rabbit which exhibited such toxic symptoms after a dose of 75 mgrms. per kilo.

While this work was in progress Graham and Poulton [1913] published a communication in which it was suggested that an important fallacy underlies the supposed creatinuria occurring in diabetes and in conditions associated with acidosis, owing to the presence of aceto-acetic acid which tends to lower the value of the preformed creatinine estimations. In order to eliminate this error in the foregoing results, several experiments were performed with special reference to the possible presence of aceto-acetic acid. A positive result was never obtained with the ferric chloride test either before or after the injection of hydrazine. Owing to the extremely unsatisfactory nature of this test in the detection of minute amounts of this acid, if any suspicious

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coloration was given with the ferric chloride solution the method recommended by Graham and Poulton was used. No trace of aceto-acetic acid was detected in any of the urines, while creatine was still found to be present in measureable amounts.

Discussion of Results.

The four experiments, in which no alteration in the sugar content of the blood, and no creatinuria, followed the injection of hydrazine, are interesting in relation to the previous work carried out by Underhill [1911].

In the present series of experiments the control estimations, carried out previous to the administration of hydrazine, show a reasonably constant sugar content of the blood, the average being approximately 0·10 per cent., thus corroborating previous investigations; while the divergence in the sugar estimations after injection appears to be dependent on the differences in susceptibility of the rabbits to the amount of the drug injected. Thus Rabbit A was used in Experiments I and VI, and Rabbit B in II and III, when 50 and 75 mgrms. per kilo. body weight were respectively administered with no resulting abnormal symptoms or creatinuria, while a dose of 75 mgrms. per kilo. had such a remarkably toxic effect on Rabbit C that it died within 12 hours of injection. After an interval of approximately one month, a larger dose was administered, 100 mgrms. per kilo. to Rabbit A (Experiment VII) and 90 mgrms. per kilo. to Rabbit B (Experiment VIII), with the result that both showed a distinct alteration in the percentage of their blood sugar, associated with the production of creatinuria. In all Underhill's experiments in which the blood sugar content was estimated, only 50 mgrms. per kilo. were administered, a dose which, in the case of the rabbits used in the present research, was never found to produce any alteration in sugar percentage. In the two other experiments he records, in which a dose of 100 mgrms. per kilo. was used, the rabbits died within five and twenty-four hours respectively, no sugar estimation being carried out. Hence it would appear that the rabbit can be relied upon to exhibit a constant hypoglycaemia after the administration of the amount of hydrazine requisite for each individual animal.

In the six experiments in which distinct creatinuria followed hydrazine injection, and in which no obvious extraneous factors could be detected, the sugar estimations show evidence of a well-marked associated hypoglycaemia. If the relationship of creatine to the total creatinine is considered, it is seen that after hydrazine injection the lowest percentage of creatine is 20·9,

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the highest being 60·3 %, whereas during the three control days previous to injection the highest figure recorded, even in the case of those rabbits which were not obtained creatine-free, is 7·2 %. The only exception was Experiment II, in which on the third day previous to injection a percentage of 16·4 was recorded. However, before administration of the drug, no creatine was obtained, while after a dose of 50 mgrms. per kilo. the relationship of creatine to total creatinine remained within what were found to be normal limits. In the two fasting experiments the $\frac{\text{creatine}}{\text{total creatinine}}$ ratio ranged from 18·2 % to 31·6 %—figures quite in accordance with previous results.

As regards the sugar content of the blood in those four experiments where the occurrence of hypoglycaemia and creatinuria were associated, before injection the figures ranged from 0·087 %–0·101 %, while after injection they lay between 0·042 % and 0·071 %, there being thus always a distinct decrease in sugar percentage beyond the limits of experimental error.

Of the two experiments in which creatinuria was not associated with the simultaneous occurrence of hypoglycaemia, no appreciable alteration in the sugar percentage figure was found in Experiment IX; while in Experiment VII, Rabbit A, in which two months previously no change in the sugar content had been produced by doses of half and three-fourths of the amount administered in the subsequent experiment, a marked hyperglycaemia resulted. To account for this contradictory result, no very satisfactory explanation can be offered. The rabbit certainly showed considerable malaise and symptomatic disturbance very shortly after receiving the injection. Considerable difficulty was experienced in obtaining the requisite amount of blood from the auricular vein, and this may have been partly due to a concentration of the blood. This possibility would in itself tend to raise the percentage figures of the sugar content; and in this connection it may also be mentioned that owing to a similar difficulty in obtaining sufficient blood from the veins in Experiment IX it was drawn directly from the heart.

It is thus evident that after the administration of a suitable dose of hydrazine a hypoglycaemia results which is invariably associated with creatinuria, while if no change in the sugar content is produced, creatine is not found in the urine. It has also to be noted that only those rabbits which showed this combined hypoglycaemia and creatinuria exhibited any marked symptoms of metabolic disturbance, the animals being quite dull and listless and refusing all food and drink.

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Of the factors which may lead to this alteration from normal metabolism, that of starvation can be eliminated on considering the results of the two fasting experiments (X and XI). Neither of the rabbits showed any reduction in the percentage of blood sugar. On the contrary, Experiment XI showed a slight increase, while in X a distinct hyperglycaemia resulted. In both cases, as already mentioned, there was a marked excretion of creatine.

Thus, although the results of the experiments here recorded point to the intimate association of carbohydrates with the metabolism of creatine, yet the exact nature of this relationship or even its degree of importance is still uncertain. It has been shown that the creatinuria following hydrazine injection is accompanied by a condition of hypoglycaemia, while it is already known that the creatinuria of diabetes and phloridzin poisoning is associated with hyperglycaemia. On the other hand, no such constant change in the sugar content of the blood is found to accompany the creatinuria induced by fasting. Furthermore, some experiments by Underhill and Rand [1910] show that "The perverted creatine metabolism as well as the metabolism of the other nitrogenous constituents of the urine tend rapidly to resume the normal on the rectal administration of dextrose, without necessarily exerting any influence on the pathological condition of the patient."

Nor do the various attempted explanations of the influence of carbohydrates on the excretion of creatine carry us very far. There is no definite evidence in favour of the hypothesis that they are essential for the conversion of creatine into creatinine or other as yet unknown substances, or for its oxidation and excretion as urea. No deductions in this connection can be drawn from previous work on the subject [MacAdam, 1913] because the creatinuria of these experiments was associated with an increased excretion of urea. So many factors enter into urea excretion, that it is impossible to eliminate them satisfactorily; and analogies drawn from *in vitro* experiments to the changes occurring *in vivo* are, in regard to the present problem, essentially untrustworthy.

That an adequate supply of carbohydrates inhibits muscle disintegration and consequently prevents the excretion of creatine is merely a special phase of the more general hypothesis suggested by Mendel and Rose [1911] that "the tissue cells may not functionate properly when the normal amount of carbohydrate food is wanting, and in this case the elimination of creatine would be analogous to the production of the acetone bodies which is also inhibited by the administration of carbohydrates." Again, Krause [1913] supports the idea that creatine may be an end product of metabolism, arguing

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that creatinuria may sometimes be not so much a deviation from normal metabolism as an indication that our conceptions of normal metabolism, based as they have been on somewhat limited observations, may not be comprehensive enough. Recent work on the creatinuria of childhood [Rose, 1911; Folin and Denis, 1913] and on its irregular occurrence during the menstrual cycle of normally healthy women [MacAdam, 1914] supports this plea. The excretion of creatine may be merely one of the earliest manifestations of an upsetting of carbohydrate equilibrium, and may occur before a slight exaggeration of an otherwise normal process has become a definitely pathological phenomenon.

Conclusions.

1. After hydrazine injection in rabbits, (*a*) if no alteration in the sugar content of the blood occurs, no marked creatinuria results; (*b*) but the excretion of creatine is associated with a condition of hypoglycaemia.

2. This creatinuria is not dependent on the starvation factor *per se*, for in the two fasting experiments the excretion of creatine was associated with an increased sugar content of the blood.

3. Creatinuria appears to be produced on the least disturbance of the equilibrium of carbohydrate metabolism, of which it is one of the earliest and most sensitive manifestations.

I desire to acknowledge my deep indebtedness to Prof. Noël Paton, and Dr E. P. Cathcart for much helpful advice and criticism during the progress of this work, which was done during the tenure of a Carnegie Scholarship.

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XX. THE FORMATION OF ELLAGIC ACID FROM GALLOYL-GLYCINE BY *PENICILLIUM*.

By MAXIMILIAN NIERENSTEIN.

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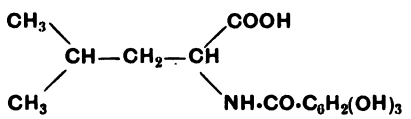
(*Received April 30th, 1915.*)

Kraemer [1900], who investigated the galls formed by *Cynips aciculata* on *Quercus cocinea*, found that during the chrysalis stage gallic acid was produced and that as the imago developed the gallic acid gave place to tannic acid. These observations gain in interest if viewed in the light of the recent investigations of v. Stockert and Zellner [1914], who have proved the presence of nitrogenous products in a number of galls caused both by insects and fungi[1], and also of the results of Nierenstein [1914] who has isolated galloyl-leucine



from the galls of *Quercus aegilops*, which are caused by *Cynips calcis*. These investigations suggest, namely, the possible formation of the digallic acid nuclei in tannic acid by way of some nitrogenous product of the galloyl-leucine type which serves as food-stuff for the growing parasites and at the same time leads to the formation of digallic acid, the presence of which in the tannic acid molecule has been established by Nierenstein [1910, 1 and 1912, 1] and which has been synthesised by Emil Fischer and K. Freudenberg [1913], so that the identification of this substance, if experimentally formed, was possible. The conditions selected as a preliminary to other investigations in progress were inquiries into the action of *Penicillium* on galloyl-glycine, which is easily prepared by the usual methods of Emil Fischer. There was, of course, the possibility, especially in the light of the beautiful investigations

[1] With reference to gall formation see E. T. Connold [1908, 1909].

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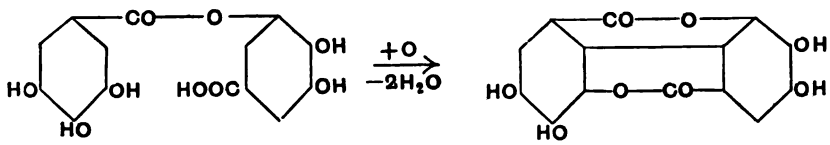
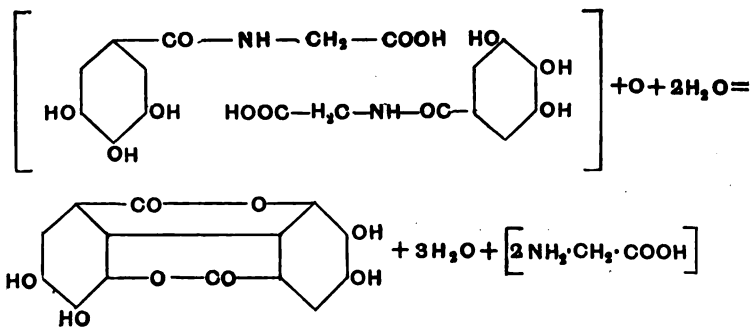
of Lewis Knudson [1913] on the action of fungi on tannic acid, that the digallic acid, if formed, would at once be decomposed into gallic acid; however, the selected conditions lead not to digallic acid, but to ellagic acid, which is a stable oxidation product of digallic acid and is frequently formed from the digallic acid nuclei of several tannins, both *in vivo* and *in vitro* [Nierenstein, 1910, 2]. It is quite possible that digallic acid is formed and subsequently converted into ellagic acid:



Digallic acid Ellagic acid

However, if one considers the above-mentioned investigations of Knudson and the pronounced oxidative properties of *Penicillium* [Herzog and Meir, 1908, 1909; Meir, 1909] it is far more likely that the elimination of the glycine radical and the ellagic acid formation through oxidation and anhydration [Nierenstein, 1912, 2] take place simultaneously:



This interpretation assumes that the glycine radical is eliminated by hydrolysis, which is hypothetical, as the present investigations have only established the formation of ellagic acid and nothing is at present known as to the fate of the glycine part.

In this connection reference is made to C. L. Berthollet [1809] and also to Merklin and Woehler [1845], who have found ellagic acid in the benzoar-stones of Persian goats, which suggests that galloyl-glycine (trihydroxy-hippuric acid) is, perhaps, also formed in the animal organism, especially as ellagic acid has also been found by Woehler [1848] in the bladder of *Castoreum canadense*.

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It must, however, be remembered that gallic acid on administration is recovered either as free gallic acid [Stockmann, 1886, 1898; Baumann and Herter, 1877; Harnack, 1898] or in combination with sulphuric acid [Rost, 1898], but never in combination with glycine [Heffter, 1905].

Experimental.

Tricarbomethoxygalloyl-glycine.

$$(CH_3—CO_2—O)_3 . C_6H_2 . CO . NH . CH_2 . COOH.$$

Five grams glycine in 75 cc. water and 3·5 g. potassium hydroxide are mechanically shaken for three hours with 26·8 g. tricarbomethoxygalloyl chloride [Emil Fischer, 1908] dissolved in ether, which is added in three portions and cooled before adding. The solution is then left for two to three hours in a cooling mixture and acidified with carefully cooled hydrochloric acid after separation from the ether. The product thus obtained crystallises from alcohol in prismatic needles, which melt at 202°–204° with decomposition and evolution of carbon dioxide. The substance is also soluble in acetone and ethyl acetate. The yield is 94 %.

In addition to the usual analysis an hydroxyl estimation by the method of Daniel and Nierenstein [1911] was also made. As in other cases, where the hydroxyls were in the meta-position to the carboxyl groups [Daniel and Nierenstein, 1911; Nierenstein, 1912, 3], nearly 2 % more carbon dioxide was formed than required by theory. This increase is derived from the carboxyl group, which is partly eliminated in these cases by the pyridine [see Bredig and Fajans, 1910; Nierenstein, 1912, 4].

Analysis. 0·2577 g. : 8·3 cc. N_2 at 19° and 767 mm.
0·1850 g. : 0·2816 g. CO_2; 0·0565 g. H_2O[1]
0·2462 g. : 0·0708 g. CO_2; (Daniel and Nierenstein)

		Calculated for $C_{15}H_{15}O_{12}N$
N	3·74 %	3·49 %
C	42·49 %	42·76 %
H	3·42 %	3·69 %
CO_2	28·86 %	26·92 %

Galloyl-glycine

$$(OH)_3C_6H_2 . CO . NH . CH_2 . COOH.$$

Five g. tricarbomethoxygalloyl-glycine suspended in water are treated on the water-bath at about 40° with 50 cc. of a 10 % solution of pyridine

[1] The combustion was carried out in oxygen [see Francis and Nierenstein, 1911].

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and water. The evolution of carbon dioxide is finished in about two hours, when the solid is crystallised from absolute alcohol. It is obtained in cubes, which melt at 282°–283°, the yield being 97–98 %. Galloyl-glycine gives like galloyl-leucine [Nierenstein, 1914] only the iron chloride reaction, but not the potassium cyanide reaction for gallic acid [Sidney Young, 1883].

Analysis. 0·2399 g. : 13·3 cc. N_2 at 18° and 755 mm.
0·1758 g. : 0·3057 g. CO_2; 0·0572 g. H_2O

		Calculated for $C_9H_9O_6N$
N	6·36 %	6·23 %
C	47·32 %	47·59 %
H	3·64 %	3·89 %

Three g. galloyl-glycine were hydrolysed with dilute hydrochloric acid (10 %) in a sealed tube and the solution extracted with ether. The ethereal part gave on evaporation gallic acid, which had the correct melting point and gave all the specific reactions for gallic acid. The aqueous solution was benzoylated; the hippuric acid obtained melted at 185°–186° and caused no depression when a mixed melting point with hippuric acid was carried out.

Formation of ellagic acid from galloyl-glycine.

Five g. galloyl-glycine and 2 g. sodium bicarbonate are dissolved in 250 cc. of water and the solution saturated with carbon dioxide. The sterilised solution is then inoculated with *Penicillium* (*spec.* ?) grown in a solution of tannic acid, which contains free sugar [see Geake and Nierenstein, 1914]. The solution becomes cloudy after standing in an incubator for 32 hours at 42° and in about 78 hours a precipitate begins to be formed. After an incubation of 22 days, the *Penicillium* is carefully removed and the precipitate collected and washed with dilute hydrochloric acid. The product gives the specific Griessmayer reaction for ellagic acid [Nierenstein, 1909] and crystallises from pyridine in prismatic needles, which contain pyridine [see Perkin and Nierenstein, 1905]. The yields of three preparations were 83 %, 87 % and 84·5 %.

For analysis the product was treated with alcohol and dried at 160°.

Analysis. 0·1946 g. : 0·3958 g. CO_2; 0·0395 g. H_2O

		Calculated for $C_{14}H_6O_8$
C	55·50 %	55·62 %
H	2·27 %	1·98 %

The acetylellagic acid prepared according to Perkin and Nierenstein [1905] crystallised from alcohol in glistening, colourless, flat needles, which

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commenced to sinter at about 331° and melted at 341°–344°, which agrees with the melting point given by Perkin and Nierenstein.

Analysis. 0·1780 g. : 0·3679 g. CO_2; 0·0489 H_2O

	Calculated for $C_{14}H_2O_6(CO{-}CH_3)_4$
C 56·37 %	56·17 %
H 3·06 %	2·98 %

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XXI. THE ACTION OF NORMAL AND BASIC LEAD ACETATE ON THE SUGARS, WITH REMARKS ON RUBNER'S TEST FOR DEXTROSE AND LACTOSE.

By HAROLD ROGERSON.

From the Chemical Department, Guy's Hospital Medical School.

(*Received May 6th, 1915.*)

During the course of some experiments on diabetic urines, Dr E. L. Kennaway observed the formation of a pink colouration in many cases when the urines had been treated with basic lead acetate and ammonia for the removal of dextrose. The colouration developed very gradually, and after two days' interval, a pink precipitate separated, leaving the solution almost colourless. As noted, the colouration was not obtained in every instance, and it was inferred either that the colouration was due to the so-called "Rubner reaction" indicating the incomplete removal of dextrose, or that some pathological substance might be present.

The author, at the suggestion of his colleague, conducted some experiments in order to elucidate this point and found that the colouration was due to dextrose.

It was deemed advisable to ascertain how other sugars behaved towards normal and basic lead acetate under different conditions, not only with and without ammonia but also other alkalies. The results obtained indicate that "Rubner's test" for dextrose and lactose is given equally well by many other sugars.

The normal lead acetate used was a saturated solution, and the basic solution was prepared according to the British Pharmacopoeia.

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I (*a*). *Normal lead acetate alone at ordinary temperature.*

1 cc. sugar solution (10 %).
10 cc. saturated lead acetate.

Dextrose	No change in 12 days.
Laevulose	Yellow in 5 days, darker in 12 days.
Galactose	Faint yellow in 1 day, gradually darker to 12th day.
Mannose	No change in 12 days.
Lactose	,, ,,
Sucrose	,, ,,
Maltose	,, ,,
Arabinose	Similar to galactose

The dextrose and galactose used in the above experiment had been soaked in alcohol for eight years, so there can be no doubt about the purity of these two sugars. If the above Table I (*a*) be compared with the Table I (*b*) it will be seen that the results depend almost entirely on the temperature. No precipitates were formed in any of the above cases.

I (*b*). *Solutions heated just to boiling.*

Dextrose	Yellow at 60°.	Orange-yellow at 100°.	No ppt.
Laevulose	,, ,,	Lighter orange-yellow.	,,
Galactose	,, ,,		Ppt.
Mannose	,, ,,		,,
Lactose	,, ,,		Very slight ppt.
Sucrose	No colour.		No ppt.
Maltose	Light yellow at 60°.	Orange-yellow at 100°.	Very slight ppt.
Arabinose	,, ,, 80°.	Darker yellow at 100°.	No ppt.

The above results indicate that the reaction alone with normal lead acetate would make it somewhat difficult to ascertain the presence of any individual sugar. Sucrose is of interest because it does not show any colour either at the ordinary temperature or on boiling the solution. This is the case also when basic lead acetate is used; indeed, sucrose is the only sugar which did not give any results which could be noted.

II (*a*). *Basic lead acetate alone at ordinary temperature.*

Dextrose	Very faint yellow in ½ hr.,	orange	in 3 days,	yellow after	18 days.
Laevulose	Light yellow	,,	yellow	,,	yellow	,,
Galactose	Orange-yellow	,,	orange	,,	orange	,,
Mannose	No change	,,	no change (slight ppt.)	,,	faint yellow	,,
Lactose	No change	,,	faint yellow	,,	yellow after	,,
Sucrose	No change	,,	no change	,,	no change	,,
Maltose	Opalescent	,,	yellowish-pink	,,	yellow	,,
Arabinose	Cf. galactose	,,	yellow	,,	yellow	,,

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II (*b*). *Solutions heated just to boiling.*

Dextrose	Faint yellow at 50°. Dark yellow at 100°. No ppt.
Laevulose	,, ,, ,, ,, Slight ppt. on cooling.
Galactose	Orange-yellow at 40°. Very dark at 70°. Lighter at 100°. Ppt.
Mannose	No colour before 100°. Yellow rapidly, then darker. Initial ppt.
Lactose	Same as mannose but no initial ppt.
Sucrose	No colour. No ppt.
Maltose	Faint yellow at 75°. Lemon-yellow at 100°, then darker.
Arabinose	Yellow at 30°. Then same as galactose.

If Tables I (*b*) and II (*b*) be compared it will be seen that the colourations obtained in the latter case are much more pronounced than those obtained in the former. The same results were observed also when the sugars were treated with the above reagents in the presence of alkalies.

III (*a*). *Normal lead acetate with ammonia.*

At the ordinary temperature, this was done with dextrose only, when the following result was obtained:

10 cc. dextrose (10 %) and 200 cc. lead acetate (7·5 %); added NH_4OH (0·880) until a slight precipitate formed which occurred after 38 cc. had been used.

After 75 minutes, a faint pink colouration was evident, which became darker until, after five days, the solution became cloudy and a copious pink precipitate separated, leaving the liquid almost colourless.

III (*b*). *Solutions heated just to boiling.*

1 cc. sugar (10 %).
1 cc. NH_4OH (0·880).
10 cc. saturated lead acetate.

Dextrose	Pink at 45°, orange-red at 100°. No immediate precipitate.
Laevulose	Yellow at 45°, darker and then lighter at 100°; again darker.
Galactose	,, ,, then same as dextrose.
Mannose	No colour before boiling, then pink to dark yellow.
Lactose	Faint yellow at 55°, darker at 100°, then reddish-brown.
Maltose	,, ,, 75°, orange ,, then opalescent.

Rubner's test for Dextrose and Lactose.

This test [Rubner, 1885] is carried out by adding some lead acetate (the ordinary laboratory reagent) to a dilute solution of dextrose, and then adding ammonia, drop by drop, until a permanent precipitate is formed.

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On standing in the cold the liquid becomes coloured gradually, first yellow and then rose or flesh red; on heating the colouration takes place more quickly. It is also stated that the addition of the reagents must take place in the order named.

A series of tests was accordingly performed according to the directions given by Rubner, when the following effects were noted:

IV.

10 cc. sugar (1 %).
1 cc. normal lead acetate (about 10 %).
NH_4OH until permanent precipitate.

On warming,	Dextrose	gave flesh colour	and precipitate.
	Laevulose	,, ,,	,,
	Galactose	,, ,,	,,
	Mannose	,, yellow colour	but no precipitate.
	Lactose	,, ,, ,,	and precipitate.
	Maltose	,, ,, to buff colour	and precipitate.

The observations, as noted in Table IV, clearly indicate the futility of adopting this reaction as a specific test for dextrose and lactose, and consequently it should be deleted from the literature.

It was deemed of interest to ascertain the effects of organic liquids on the coloured solutions and precipitates obtained in the above-described reactions. On shaking the solutions, in which a colouration had developed, with ether or chloroform the colour disappeared; but, on keeping, the colouration was gradually restored, and on renewed shaking did not entirely disappear.

The effect of using alcohol was then tried. A saturated 90 per cent. alcoholic solution of dextrose was treated with alcoholic solutions of lead acetate and ammonia, when a colouration ranging from pink to orange-red was obtained on standing at the ordinary temperature. The precipitate first formed dissolved in the excess of lead acetate solution added, but no precipitate was subsequently thrown down, and the colour was confined solely to the solution.

The behaviour of basic lead acetates towards the sugars is much more sensitive than that of the normal salt, and the experiments to be described have been extended to most of the sugars one would be likely to meet with in biochemical investigations. Other alkalies besides ammonia were tried, as well as acetates of other metals, but in the latter case no definite effects were observed.

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V (*a*). *Dextrose, basic lead acetate and ammonia at the ordinary temperature.*

10 cc. dextrose (10 %).
5 cc. NH_4OH (0·880).
100 cc. basic lead acetate (B.P. fortis).

After 2 minutes	Yellow tint	Solution clear
1 hour	Salmon pink	,, ,,
2 hours	Blood-red	,, ,,
3·5 ,,	Darker red	,, ,,
21·5 hours	Very dark red	,, ,,
3 days	Lighter red	Pink precipitate
14 ,,	Dark yellow	Pink and buff precipitate

It will be seen from the above Table V (*a*) that the colour goes to a maximum before any precipitate forms; and it was also observed by using 10, 5, and 1 per cent. solutions that the amount of precipitate actually formed on mixing the solutions was proportional to the amount of sugar present, as was also the maximum tint developed. Therefore the colour is proportional to the sugar-strength of the solution. Other sugars and polyhydric alcohols gave the following results:

V (*b*).

Laevulose (m.p. 100)	Same as dextrose	Pink precipitate
Galactose (m.p. 144)	Same as dextrose	Pink ,,
Mannose	No colour	White cryst. precipitate
Lactose	Reddish-orange	Pink precipitate
Maltose	Yellow	Buff ,,
Sucrose	No colour	No ,,
Arabinose	Dark orange	Buff ,,
Xylose	Slight pink	Pink ,,
α-Methyl glucoside	No colour	White cryst. precipitate
Dulcitol	,,	White amorph. ,,
Mannitol	,,	No precipitate

The results shown in the above Table V (*b*) indicate that it would be almost impossible to identify any particular sugar tested under the above conditions at ordinary temperature. A full series of observations were then made with various sugars, the solutions being raised to boiling, and the temperature noted at which any colouration first appeared.

The following table shows the results obtained for dextrose.

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V (*c*).

Experiment	Dextrose (10 %) in cc.	H_2O in cc.	$NH_4 \cdot OH$ (·880) in cc.	Basic lead acetate in cc.	Solutions before heating	Effect on heating
1.	1·0	0·0	1·0	10·0	Clear after few seconds	Pink at 45°. Then darker.
2.	0·5	0·5	1·0	10·0	Clear at once	,, 55° ,, ,,
3.	0·1	0·9	1·0	10·0	,, ,,	Pink at 65°. Then yellow and white cryst. ppt. slowly forms.
4.	0·1	0·9	0·5	5·0	,, ,,	Pink at 63°. Darker yellow than (3).
5.	0·1	0·9	0·1	0·9	,, ,,	Pink at 62°. Darker than (3) or (4), but does not turn yellow.
	(1 %)					
6.	1·0	0·0	1·0	10·0	,, ,,	Pink at 65°. Then yellow (cf. 3).
7.	0·5	0·5	1·0	10·0	,, ,,	Pink at 75°. Yellow at 80°, white cryst. ppt. on standing.
8.	0·1	0·9	1·0	10·0	,, ,,	No colour. White cryst. ppt. on boiling. Almost solid.
9.	0·0	0·0	1·0	10·0	,, ,,	Same as 8.
10.	0·1	0·9	0·5	5·0	,, ,,	No colour. White cryst. ppt. on boiling, but not large.
11.	0·1	0·9	0·1	0·9	,, ,,	Yellow at 95°. Slight ppt.

The above results in Table V (*c*) clearly indicate the sensitiveness of the reaction. The white crystalline precipitate obtained in 8, 9, and 10 is of interest since this is known to be a basic lead acetate of definite composition, and the fact that it is only formed when the sugar is present only in very small amount shows that dextrose (and other sugars) exerts an inhibitive effect on its formation.

A corresponding series of experiments was performed with all the other sugars and the results obtained were much of the same order. Only one test with each is quoted in the following table, the quantities used being the same as in the first experiment in Table V (*c*).

VI.

Sugar	Solution before heating	Effect on heating
Laevulose	Solution not quite clear	Yellow, then pink, clear at 70°, then dark orange on boiling.
Galactose	Solution clears slowly	Yellow at 30°, orange-pink at 65°, pink ppt. separates.
Mannose	,, ,, ,,	No colour before boiling, then yellow. No pink, orange or ppt.
Lactose	Considerable ppt.	Faint yellow at 60°, yellow on boiling. Rapid pink ppt. Solution orange.
Maltose	,, ,,	Opalescent on warming, yellow on boiling, then pink, and orange. Pink ppt.
Sucrose	Solution clears slowly	No colour and no ppt.

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In describing his test, as previously noted, Rubner directs the reagents to be added in a definite order, viz., to the sugar solution add lead acetate and then ammonia. The above experiments, however, show that it is quite immaterial whether the lead solution or the ammonia is added first, the difference being that, if the ammonia is added first, the initial precipitate formed on adding the lead solution is much greater than if the solutions are mixed in the alternative order. In any case the result is the same.

Basic lead acetate and different alkalies with dextrose.

It was deemed of interest to see how other alkalies would behave, and the hydroxides of sodium and barium were accordingly tried with dextrose in the presence of basic lead acetate. The results, which were obtained at the ordinary temperature, may be thus summarised:

VII.

NaOH	After 15 minutes, white precipitate.
	After 50 minutes, slight pink colouration evident.
	After 15 hours, orange. No more precipitate.
$Ba(OH)_2$	Fugitive yellow which became permanent after 7 hours.

The action of different acetates with dextrose and alkali.

Since lead acetate gives intense colourations with many sugars, it was decided to see if the acetates of other metals gave similar reactions. The acetates of the following metals were used: barium, zinc, mercury, manganese, cadmium and uranium. Of these, the only acetate which showed any colouration was that of barium, when the solution was at the ordinary temperature. In this case the following results were obtained:

VIII.

$Ba(C_2H_3O_2)_2$ and $Ba(OH)_2$	After 1 day, yellow colour, no precipitate.
„ „ NH_4OH	„ 4 days, faint yellow, no precipitate.

The action of alkalies alone on the sugars.

One of the characteristic tests for dextrose is the action of sodium or potassium hydroxide (Moore's reaction), and barium hydroxide behaves similarly. Therefore in performing the tests described above where NaOH and $Ba(OH)_2$ were used, the effects observed at the ordinary temperature have been noted. Moreover, the present author has observed that if solutions of various sugars are boiled with ammonia for a short time, colourations

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varying from pink to yellow are formed. The following results were obtained with 10 per cent. solutions, and boiling for a few minutes with about an equal volume of ammonia (0·880).

IX.

Dextrose	Faint yellow in 1 minute. Distinct in 3 minutes.
Laevulose	" " " " "
Galactose	" " " " "
Mannose	Very faint yellow after 3 minutes.
Lactose	Cf. dextrose. Yellowish-pink on standing.
Maltose	Faint yellow in 1 minute. Orange in 3 minutes.
Arabinose	No change.

It will be apparent from the above observations that the combined action of the acetates of lead and ammonia is somewhat complex, and it is impossible to correlate the effects observed with any particular sugar in order to identify it. No quantitative experiments have been carried out up to the present, but there is little doubt that the precipitates obtained in many cases are complex derivatives of the sugar and the acetates of lead.

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XXII. THE QUANTITATIVE DETERMINATION OF GLYOXALASE IN THE BLOOD AND SOME OF ITS APPLICATIONS.

By HAROLD WARD DUDLEY.

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(*Received May 8th, 1915.*)

In previous communications [Dakin and Dudley, 1913, 1914] the properties of the enzyme glyoxalase, which converts α-ketonic aldehydes into α-hydroxy-acids according to the general scheme:

$$R . CO . CHO + H_2O = R . CHOH . COOH$$

have been discussed. It has been demonstrated that the enzyme, which is widely distributed, rapidly converts methylglyoxal into lactic acid, and is therefore most probably of importance in the intermediary metabolism of carbohydrates. In order to investigate more closely some problems connected with the enzyme a quantitative method of estimation was desirable, and the object of the present paper is to describe such a method and some of the results obtained by its aid.

On account of the difficulty of the quantitative extraction of lactic acid from liquids containing protein and the laboriousness of its determination, methylglyoxal could not be used with any hope of success as substrate in a quantitative method for estimating glyoxalase activity. The previous qualitative work on the enzyme was greatly facilitated by the employment of phenylglyoxal as substrate. The ease with which the optically active mandelic acid produced by the action of the enzyme can be extracted from digestion mixtures containing protein and the high value of the specific rotation of the acid, render quick determinations of the activity of glyoxalase preparations possible. Accordingly a quantitative method was devised, using phenylglyoxal as substrate, which was in principle essentially the same as the method employed in previous qualitative work on the enzyme.

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The primary object of the quantitative estimation of glyoxalase was the comparison of its activity in the living animal under varying conditions in the hope of gaining more detailed knowledge as to its function in carbohydrate metabolism. Blood was therefore chosen as the tissue in which to determine the activity of the enzyme, since its withdrawal from time to time in small quantities is without effect on the general condition of the animal, and comparisons between equal volumes of different specimens can be accurately made.

The method consists in allowing 5 cc. of defibrinated blood to act on phenylglyoxal in the presence of calcium carbonate at 37° for 20–24 hours. The protein is then precipitated by adding solid ammonium sulphate and heating. The mixture, after acidification, is filtered, and the protein precipitate is washed free from mandelic acid, which is then extracted from the filtrate with ether and determined polarimetrically.

It was found at the outset that the concentration of phenyglyoxal is by no means a matter of indifference. It exhibits a toxic effect on the enzyme in concentrations even as low as 0·80 %. It is therefore necessary to have a standard concentration in all quantitative determinations. It was further shown that the method is not applicable without modification to amounts of blood greater than 7 cc., mainly because of the difficulty of washing the bulky protein precipitate free from mandelic acid. Amounts of blood from 1 to 7 cc. give amounts of active mandelic acid, measured polarimetrically, in simple proportion to the quantity of blood taken.

By means of this method the fact has been established that the "glyoxalase value" for the blood of different animals of the same species is remarkably constant, and is also definite for the particular species. Comparisons between species show definite differences in the glyoxalase values of the blood. In descending order of glyoxalase activity the species so far examined arrange themselves thus:

Dog, Ox, Rabbit, Sheep, Pig.

The order in which these animals appear is interesting. There appears to be a rough parallelism between the glyoxalase activity and the sugar tolerance of the animals. It has been shown by Hunter and Hill [1914] that "the sheep's capacity to deal with subcutaneously administered glucose is greatly inferior to that of the dog," while Carlson and Drennan [1912] have observed an extraordinarily low sugar tolerance in the case of the pig. It will be noticed that the sheep and the pig show the lowest values for blood

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glyoxalase in the various species so far examined, whilst the value for the dog is the highest. Experiments of Underhill and Closson [1906] have demonstrated that the dog can utilise large quantities of glucose introduced parenterally without developing a glycosuria. They have shown, too, that the rabbit is able to assimilate much glucose injected subcutaneously; indeed under the experimental conditions observed by them its sugar tolerance appears to be similar to that of the dog. But apparently no accurate determinations of the limits of sugar tolerance in these animals have been made. If the glyoxalase of the blood is any guide to the sugar tolerance of an animal one would expect the rabbit to occupy an intermediate position between the dog and the sheep. It certainly can assimilate more parenterally administered glucose than the sheep, but its efficiency as compared with the dog has not been determined.

Without doubt the sugar tolerance of an animal must be governed by the interplay of many complicated factors, and of these glyoxalase activity may be one, so that the apparent parallelism between the sugar tolerance of an animal and the glyoxalase value of its blood may not be entirely fortuitous.

Experiments on the glyoxalase activity of the blood in various forms of glycosuria are in progress and will be reported shortly.

Experimental.

Quantitative determination of glyoxalase in 5 cc. of blood.

Before the blood is shed a 250 cc. conical flask containing 10 cc. of a suspension of 2·75 g. chalk in 100 cc. water is placed in an incubator at 37°. A 1·2 % solution of phenylglyoxal hydrate is also heated to 37°. The experimental animal is then bled and the blood is whipped until free from fibrin, when it is strained through muslin. 10 cc. of the phenylglyoxal solution (0·12 g. phenylglyoxal hydrate) are then well mixed with the chalk suspension and 5 cc. of the blood are finally added. It is important to make the additions in this order since most phenylglyoxal preparations give distinctly acid solutions. This acidity is removed by the chalk before the blood is added and so can do no damage to the glyoxalase, which has been shown to be very sensitive to acid. The mixture is then incubated at 37° for 20–24 hours. Experiments have been performed which show that the action is complete in this period. Phenylglyoxal displays distinctly antiseptic properties, so that the addition of toluene is unnecessary. In no case in the performance of many experiments has any bacterial activity been

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observed. After completion of the reaction 25 g. ammonium sulphate are added to the mixture, which is then heated in a boiling water-bath for 3–4 minutes. The protein is thereby precipitated. After cooling, 12 cc. of 50 % phosphoric acid are added and the liquid is allowed to stand, with frequent shaking, for half an hour. The protein precipitate is then filtered on a Buchner funnel and washed five times with 10 cc. 40 % ammonium sulphate solution. These washings must be performed slowly, each washing being allowed to soak well into the precipitate before being sucked through. A water-clear filtrate is obtained which is extracted four times with small quantities of ether (10–12 cc.). Each ether layer is washed with distilled water (about 3 cc.). The mandelic acid is completely removed by this process. The ether is then removed by distillation and a crystalline residue of mandelic acid is obtained. This is dried in a desiccator over sulphuric acid and then dissolved in 12 cc. distilled water, solution being accelerated by warming on a water-bath. A pinch of animal charcoal is added to the hot solution, which is first allowed to cool and is then filtered. The small amount of fat which usually appears in the ether residue is completely removed by these means. The solution is then examined polarimetrically in a 2 dm. tube, the reading giving a measure of the amount of *l*-mandelic acid formed, and so of the glyoxalase content of the blood.

Experiments to show the effects of increasing concentration of phenylglyoxal and of dilution, and the completeness of removal of mandelic acid from the protein precipitate.

Sheep's blood was used in this series. The technique was that described in the standard method outlined above.

Expt.	Chalk cc.	Water cc.	Phenyl-glyoxal cc.	Blood cc.	Total vol. cc.	α	*l*-mandelic acid, g.
1.	10	30	5 (0·1 g.)	5	50	−0·66°	0·0251
2.	10	25	10 (0·2 g.)	5	50	−0·62°	0·0235
3.	10	25	10 (0·2 g.)	5	50	−0·63°	0·0239
4.	10	15	20 (0·4 g.)	5	50	−0·45°	0·0171
5.	10	75	10 (0·2 g.)	5	100	−0·60°	0·0228

After the usual filtration and washing of the precipitate in Experiment 2, the protein was subjected to five further washings by 10 cc. 40 % ammonium sulphate solution and these washings were extracted four times with ether. No mandelic acid was obtained by this treatment, showing that the acid had been completely removed by the previous manipulations.

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Experiments 2 and 3 serve to demonstrate how closely duplicate determinations agree.

Experiment 4 shows definitely the toxic effect of higher concentrations of phenylglyoxal on the enzyme.

Experiment 5 shows that dilution of the reaction mixture to twice the volume taken in standard experiments is without appreciable effect on the result.

Experiments showing the applicability of the method for amounts of blood from 1 *to* 7 cc. *and its failure for larger quantities.*

The first series was carried out with ox blood, the second with sheep's blood. I am indebted to Dr H. E. Woodman for the second series.

OX BLOOD.

Expt.	Chalk cc.	Water cc.	Phenyl-glyoxal cc.	Blood cc.	α	*l*-mandelic acid, g.
1.	10	29	10 (0·2 g.)	1	−0·19°	0·0072
2.	10	26	10 (0·2 g.)	4	−0·88°	0·0334
3.	10	25	10 (0·2 g.)	5	−1·04°	0·0395
4.	10	25	10 (0·2 g.)	5	−1·03°	0·0391
5.	10	23	10 (0·2 g.)	7	−1·31°	0·0497
6.	10	20	10 (0·2 g.)	10	−1·06°	0·0403
7.	10	15	10 (0·2 g.)	15	−0·84°	0·0319
8.	10	10	10 (0·2 g.)	20	−0·56°	0·0213
9.	Protein precipitate of 8, further washed with 50 cc. 40 % ammonium sulphate solution, filtrate extracted with ether: a quantity of mandelic acid was extracted which, examined under standard conditions, gave $\alpha = -0{\cdot}40°$.					

SHEEP'S BLOOD.

Expt.	Chalk cc.	Water cc.	Phenyl-glyoxal cc.	Blood cc.	α	*l*-mandelic acid, g
1.	10	29	10 (0·1 g.)	1	−0·12°	0·0045
2.	10	27	10 (0·1 g.)	3	−0·35°	0·0133
3.	10	25	10 (0·1 g.)	5	−0·63°	0·0239
4.	10	23	10 (0·1 g.)	7	−0·79°	0·0300
5.	10	21	10 (0·1 g.)	9	−0·57°	0·0216

Experiments 1 to 5 of the first series and 1 to 4 of the second show that the amount of *l*-mandelic acid produced is proportional to the amount of enzyme taken. The effect of adsorption of mandelic acid by the protein precipitate is beginning to be felt when 7 cc. of blood are taken, and with higher quantities the results are of no value.

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Experiments showing the rate of the reaction under the conditions of the standard determination.

A series of experiments, using 5 cc. of dog's blood in each, carried out according to the standard method, were interrupted at various intervals and afforded information with regard to the progress of the reaction.

Action stopped after	1	2	4	6	10	20	23 hours
$\alpha =$	$-0{\cdot}28$	$-0{\cdot}39$	$-0{\cdot}57$	$-0{\cdot}67$	$-0{\cdot}83$	$-0{\cdot}99$	$-0{\cdot}99°$

It will be seen that the velocity of the reaction is large at first, diminishing considerably in the later stages, and that under the conditions of experiment the reaction is complete in 20 hours.

Normal glyoxalase values for various animals.

5 cc. of freshly-shed, defibrinated blood were used in all these determinations, which were carried out in accordance with the standard method.

Dog. The following rotations for *l*-mandelic acid obtained by the action of 5 cc. dog's blood in the cases of seven different animals, most of the experiments being performed in duplicate, serve to show how constant is the value of the glyoxalase content of the blood in a particular species. The dogs, some male and some female, were of different breeds.

Dog	1	2	3	4	5	6	7
$\alpha =$	$\begin{cases} -1{\cdot}05° \\ -1{\cdot}04° \end{cases}$	$-1{\cdot}04°$	$\begin{cases} -1{\cdot}02° \\ -1{\cdot}03° \end{cases}$	$\begin{cases} -1{\cdot}08° \\ -1{\cdot}00° \end{cases}$	$\begin{cases} -1{\cdot}07° \\ -1{\cdot}06° \end{cases}$	$\begin{cases} -1{\cdot}03° \\ -1{\cdot}03° \end{cases}$	$-1{\cdot}05°$

Mean value for $\alpha = -1{\cdot}04° = 0{\cdot}0395$ g. *l*-mandelic acid.

Ox. The blood of two animals was investigated.

Mean value for $\alpha = -1{\cdot}03° = 0{\cdot}0382$ g. *l*-mandelic acid.

Rabbit. Four animals were used.

Mean value for $\alpha = -0{\cdot}74° = 0{\cdot}0281$ g. *l*-mandelic acid.

Sheep. Duplicate determinations were made on the blood of two animals.

Mean value for $\alpha = -0{\cdot}63° = 0{\cdot}0239$ g. *l*-mandelic acid.

Pig. Two animals were investigated.

Mean value for $\alpha = -0{\cdot}61° = 0{\cdot}0232$ g. *l*-mandelic acid.

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Summary.

A method for the quantitative determination of the glyoxalase activity of blood is described.

It is shown that the glyoxalase of the blood is remarkably constant in different animals of the same species under normal conditions. It is further demonstrated that different species display definite differences in the glyoxalase activity of the blood, and attention is drawn to the observation that animals displaying high glyoxalase values are those having high sugar tolerance, whilst those with lower glyoxalase activities have less well developed powers of assimilating sugar.

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XXIII. THE FORMATION OF HUMIC BODIES FROM ORGANIC SUBSTANCES.

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(*Received May 10th, 1915.*)

The true nature of the dark brown and black substances formed by the decomposition of organic matter in the soil, and grouped together under the collective name of humus, has long been a problem to both chemists and biologists, owing to the fact that the composition of these substances varies not only with the nature of the humus-producing materials, but also with the temperature, moisture and pressure relations, and the methods of extraction.

A study of the extensive literature which deals with humus shows that the widely divergent opinions expressed and the inconsistent results obtained group themselves around two chief views regarding the nature of humus.

The first view is that humus has a simple composition and consists of one or a few definite chemical substances. De Saussure [1804] describes it as a "brown combustible powder soluble in alkalies and ammonia compounds." Mulder [1849] says that it consists of seven different organic substances—crenic acid, apocrenic acid, geic acid, humic acid, humin, ulmic acid and ulmin. He considered these substances to be intimately related, and five at least were distinct steps in the decay of organic matter in the soil.

This view of the simple composition of humus was the dominant one amongst the earlier writers on the subject; and we find it existing even to-day in a slightly modified form in most agricultural text-books. In these books humus is regarded either as a more or less definite substance which can be obtained by extracting the soil with a dilute alkali after first treating with a dilute acid to remove the lime which renders the humus insoluble, or as being synonymous with the so-called humic acid which can be precipitated from alkaline solutions of the soil.

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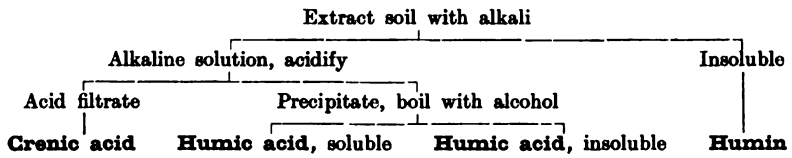
The second view, that humus is of a very complex nature, was first advanced by Hermann [1842]. He laid special stress on "the presence of a great number of various kinds of humus-substances," and divided these various substances into three groups: *humus extract*, soluble in water; *humic acid*, soluble in alkalies and precipitated by acids; *humin*, insoluble in water and alkalies.

Hoppe-Seyler [1889] added another group which he calls the *phlobaphene* group, comprising those substances dissolved out by alcohols from the acid precipitates of the humic acid group.

Still more recently Schreiner and Shorey [1910] have given a somewhat similar grouping for the various compounds they have isolated from the organic matter of soils. They, however, include another group—the *crenic acid* group—comprising the substances isolated from the acid filtrate obtained when an alkaline solution of the soil is treated with mineral acid. Their grouping may be illustrated as follows:



Schreiner and Shorey have isolated and identified 20 distinct compounds from the organic matter of soils, only one of which—a pentosan—is a carbohydrate. These substances have all been obtained from the crenic acid and alcohol-soluble humic acid groups, and nothing is known as to the identity of the constituents of the humin and alcohol-insoluble humic acid groups. Yet they state that these last two groups, the components of which are unknown, contain 39·8 per cent. of the total organic carbon found in soils.

When one considers that carbohydrates, represented by sugars, starch and celluloses, make up by far the greater portion of the organic substance of plants and so form the greater part of the material from which soil humus is derived, it appears strange that so far only one carbohydrate has been isolated from soil, and the suggestion naturally arises that there may be some connection between carbohydrates and these unknown components of the humic acid and humin groups.

The probability of such a connection was indicated during a research as to the value of certain kinds of so-called "humates" as a nutrient for the nitrogen-fixing organism *Azotobacter*, and the present paper describes the results of an investigation of the relationship between these substances.

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It has been pointed out that the terms humic acid and humin are used with varying connotation by different writers. For the purpose of this investigation it has been assumed that the terms refer to two groups of humic substances: *humic acid*—substances thrown down as brown colloidal precipitates by mineral acids from the water or alkaline extracts of humus; *humin*—substances insoluble in water and alkalies, but rendered soluble by fusing with caustic soda or potash, from the solution of which humic acid can again be precipitated.

Humic Bodies from Sugars by the action of Mineral Acids.

The discovery by Malaguti [1858] that humic acid and humin are formed when sugar is boiled with either hydrochloric or sulphuric acid was important as indicating a possible origin for these substances in the soil, but no attempt was made to trace their inter-relationship.

Later, Conrad and Guthzeit [1885] stated that on boiling raw sugar with dilute hydrochloric acid it is first changed into dextrose and laevulose, and that then the dextrose forms humic acid and the laevulose humin. As these authors give no experimental proof of the accuracy of their statement, an experiment was made to test the effect of boiling sucrose, dextrose and laevulose separately with hydrochloric acid. Five grams of each were boiled with a 7·5 per cent. solution of hydrochloric acid under a reflux condenser for 30 minutes. All three yielded a brown deposit which on testing proved, in each case, to be a mixture of humic acid and humin. The relative proportions, however, of these two substances in the three mixtures showed considerable variation, and the proportion of humic acid to humin always diminished as the boiling continued. It was found that with a strong solution of hydrochloric acid the conversion of humic acid into humin took place rapidly. In order therefore to trace in detail the changes taking place, a weak solution of the acid was employed. Five g. of sucrose, dextrose and laevulose respectively were boiled with a 3 per cent. solution of hydrochloric acid under a reflux condenser for varying periods of time. As is well known, the colourless solutions on heating pass through a series of colour changes: yellow, orange, red, brown, black, and with the brown colour the formation of a deposit is first noticed. Laevulose and sucrose pass through these changes fairly rapidly, but with dextrose the rate of change is much slower.

Laevulose:	Yellow 1 minute;	red 5 minutes;	brown 8 minutes.
Sucrose:	„ 2 minutes	„ 8 „	„ 12 „
Dextrose:	„ 11 „	„ 60 „	„ 90 „

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The first deposit obtained in each case was found to be humic acid without a trace of humin. On further boiling the humic acid becomes changed into humin. The proportions obtained by boiling for periods of 0·25, 0·5, 1, 2 and 3 hours are given in the following table:

		15 mins.	30 mins.	1 hr.	2 hrs.	3 hrs.
5 g. sucrose;	total ppt.	0·106	0·077	0·140	0·254	0·405
,,	humic acid	0·007	0·040	0·101	0·148	0·157
,,	humin (by diff.)	0·009	0·037	0·039	0·106	0·248
5 g. dextrose;	total ppt.	No deposit	No deposit	No deposit	0·003	0·011
,,	humic acid				0·002	0·005
,,	humin (by diff.)				0·001	0·006
5 g. laevulose;	total ppt.	0·028	0·156	0·244	0·492	0·685
,,	humic acid	0·010	0·103	0·104	0·184	0·165
,,	humin (by diff.)	0·018	0·053	0·140	0·308	0·520

From the above results it is seen that as the boiling is prolonged the proportion of humic acid to humin decreases, although the total amount of the deposit increases. This suggests that humic acid is an intermediate stage in the conversion of sugars into humin. That humic acid is directly convertible into humin was proved by boiling freshly precipitated humic acids, obtained from peat and laevulose, with a 7·5 per cent. solution of hydrochloric acid for four hours, when it was found that 3·5 per cent. of the peat acid and 98 per cent. of the laevulose acid had been converted into humin.

The varying proportions of humic acid and humin in these deposits from sugar probably account for the diverse analytical results which have been obtained by various investigators.

A comparison of the so-called "artificial" humic acid from sugar with the "natural" product from soil or peat shows that in appearance, solubility and behaviour towards alkalies they are almost identical. Yet when analysed a considerable difference is seen to exist in their composition.

Robertson, Irvine and Dobson [1907] state that the average composition of different specimens of artificial and natural humic acids obtained by an ammonium salt was as follows:

	C	H	O
Artificial (sugar) humic acid	64·74	4·69	29·81
Natural (peat) ,,	54·29	4·94	38·16

This variation in composition leads these authors to consider that a structural difference exists between the artificial and natural acids.

Recent researches by Baumann [1909] have shown that natural humic acid freshly precipitated possesses colloidal properties, especially the power of forming adsorption compounds.

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Considering that natural humic acid is formed in association with various decomposition products, it is probable that the true humic acid substances formed from the carbohydrates of the soil are admixed with various other products. As already indicated, some of these bodies have been extracted by means of alcohol from natural humic acid, and it was considered probable that, if the theory is correct that carbohydrates are the primary source of humic acid in the soil, the composition of the natural humic acid after purification by alcohol would approximate more closely to that of artificial humic acid.

This hypothesis was confirmed as follows: a specimen of "natural" humic acid was obtained by treating finely divided peat with a 4 % solution of hydrochloric acid until all soluble salts were extracted and then digesting the well-washed residue with an excess of 5 % ammonium hydrate solution. The dark brown liquid obtained after filtering the mass on a Buchner funnel was acidified with strong hydrochloric acid, and the flocculent brown precipitate thus obtained was separated by filtration and well washed. It was then redissolved in ammonia and reprecipitated by hydrochloric acid, the precipitate being washed with distilled water until free from chlorides. The residue was divided into two portions, one of which was dried in the steam-oven at 100°, while the other was boiled for one hour under a reflux condenser with absolute alcohol. The alcohol was filtered off, the residue washed with fresh alcohol, and dried. The average results of a series of combustion analyses of these two products were as follow:

	C	H	O (by diff.)
Natural humic acid	48·64	4·55	46·81
Natural humic acid after extraction with alcohol	60·37	5·39	34·24

These results indicate that when natural humic acid has been purified by means of alcohol its carbon content approximates much more closely to that of the artificial acid, the alcohol having removed most of the adsorbed bodies. It is well known that the carbon content of artificial humic acid varies with the method of its preparation, and no definite formula can be assigned to it, but it was found that the above analysis of purified natural humic acid showed a remarkable similarity with that of one of the artificial humic acids we obtained by boiling dextrose with hydrochloric acid. This is shown by the following comparison:

	C	H	O (by diff.)
Artificial humic acid	60·74	5·13	34·13
Natural humic acid (purified)	60·37	5·39	34·24

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Humic Bodies from Sugars by the action of Organic Acids.

It is well known that the humic acid and humin groups of substances are formed during the decomposition of organic matter in the soil. It is also known that in the process of this decomposition various organic acids are produced by bacterial activities. Experiments were therefore made to determine the effect on sugars of certain organic acids known to exist in the soil.

It was found that lactic, acetic, propionic, butyric, citric, tartaric and oxalic acids when boiled with either sucrose, dextrose or laevulose yield humic acid and humin, after producing the characteristic series of colour changes obtained by the action of mineral acids on sugars. The rapidity of the action varies with the kind and concentration of acid used, as shown by the following tables:

The action of 12·5 % *acids on sucrose.*

Acid	Time for yellow colour	Time for red colour	Time for first deposit	Wt. of total ppt. in 24 hrs.	Wt. of re-ppd. humic acid in 24 hrs.
Lactic	1 hr. 15 mins.	2 hrs.	5 hrs. 10 mins.	0·123 gm.	0·082 gm.
Acetic	2 hrs.	7 hrs. 10 mins.	12 hrs. 10 mins.	0·050 gm.	0·025 gm.
Propionic	3 hrs.	9 hrs. 10 mins.	15 hrs. 30 mins.	0·016 gm.	0·009 gm.
Butyric	3 hrs. 10 mins.	12 hrs. 10 mins.	20 hrs.	0·024 gm.	0·004 gm.

The action of 36 % *acids on sucrose.*

Acid	Time for yellow colour	Time for red colour	Time for first deposit	Wt. of total ppt. in 24 hrs.	Wt. of re-ppd. humic acid in 24 hrs.
Lactic	Immediate	2 mins.	5 mins.	0·715 gm.	0·695 gm.
Acetic	5 mins.	35 mins.	10 hrs.	0·211 gm.	0·200 gm.
Propionic	Immediate	12 mins.	16 hrs.	0·365 gm.	0·164 gm.
Butyric	55 mins.	4 hrs. 40 mins.	18 hrs.	0·040 gm.	0·008 gm.
Citric	20 mins.	1 hr.	2 hrs. 40 mins.	0·042 gm.	0·024 gm.
Tartaric	10 mins.	15 mins.	30 mins.	0·009 gm.	0·009 gm.
Oxalic	Immediate	5 mins.	25 mins.	0·160 gm.	0·093 gm.

Humic Bodies from Sugars by the action of Heat.

In the course of the present investigation it was found that the action of heat alone on sucrose, dextrose and laevulose will produce bodies very similar to humic acid and humin.

It is well known that sucrose fuses at 160°, and is converted into a mixture of dextrose and laevulosan; on further heating to about 190° it yields caramelan and at higher temperatures gradually becomes carbonised.

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In a preliminary experiment it was found that the black mass produced by heating sucrose at 220° for two hours contained at least four distinct substances: (1) water-soluble caramelan; (2) a water-soluble substance precipitated as fine particles by hydrochloric acid; (3) an alkali-soluble substance giving the typical flocculent precipitate of humic acid when treated with hydrochloric acid; (4) a black residue insoluble in water and alkalies giving the characteristic humin reactions when fused with caustic soda or potash.

Further experiments showed a great difference between dextrose and laevulose in their heat reactions, laevulose forming a black mass at a much lower temperature than dextrose. Dextrose on heating for four hours at 180° forms a light brown toffee-like mass completely soluble in water, but giving no precipitate with hydrochloric acid. On further heating for two hours at 220° it changes to a dark brown colour. Very little of this is water-soluble. The residue is almost entirely soluble in a 5 per cent. soda solution, and gives no trace of a precipitate with hydrochloric acid. On heating for three hours more at 280° it is converted into a black brittle mass, insoluble in both water and alkalies, but yielding humic acid on fusing with caustic soda and precipitating with hydrochloric acid. This black mass is evidently very similar to humin.

Laevulose even at the low temperature of 120° forms water-soluble substances which give a granular precipitate with hydrochloric acid if the heating be continued long enough, about six hours. When heated for two hours at 150° it gives a dark chocolate-brown mass all of which is soluble in water, giving a typical flocculent humic acid precipitate. Heated for two hours more at 180° it forms chiefly alkali-soluble humic acid, and further heating at 200° for two hours converts a large proportion of this into humin.

Here again, as in the acid-formed substances, humic acid is antecedent to humin. One important point of difference, however, must be noted between the acid-formed and heat-formed humic acid. The heat-formed humic acids after being dried in a water bath at 100° are no longer soluble in dilute alkali solutions; only on fusing with caustic potash or soda are they rendered soluble.

Humic Bodies from Proteins.

Proteins are said on hydrolysis to yield "an insoluble brownish black residue of humin." A careful examination of this black residue shows that it is chiefly composed of humic acid and contains very little humin. That

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the formation of this humic acid depends on the amount of carbohydrate present in the protein is seen by comparing the amount of humic acid obtained from proteins with varying content of carbohydrate. Mucin and ordinary egg white are rich in carbohydrates; caseinogen and purified egg white are practically free from carbohydrates. One gram of each of these substances, the dry weight of the ordinary egg white being calculated by a moisture determination, was boiled for eight hours under a reflux condenser with 33 cc. of a 7·5 per cent. solution of hydrochloric acid. The contents of each flask were filtered, the residue extracted with a 5 per cent. solution of caustic soda, and the extract acidified with hydrochloric acid. The precipitate of humic acid was filtered, washed, dried and weighed, with the following results:

Mucin	0·021 g.	Caseinogen	0·001 g.
Ordinary egg white	0·035 g.	Purified egg white	no precipitate.

A similar experiment with tyrosine and asparagine gave no trace of humic acid after eight hours' boiling.

Conclusions.

The general result of the investigation so far has been to indicate that carbohydrates generally and certain sugars in particular pass through a regular series of changes when submitted to the reactions described. It was also found that air-dried *sphagnum* moss is readily acted on by acids, with the formation of a brown peat-like mass from which humic acid can be extracted.

One hundred grams of air-dried *sphagnum* moss when boiled for 24 hours with 5 per cent. solutions of hydrochloric, oxalic and lactic acids respectively yielded the following results:

Hydrochloric acid	11·7 g. humic acid
Oxalic acid	2·3 g. ,,
Lactic acid	1·8 g. ,

The humic substances comprising the humic acid and humin groups probably pass through a series of changes characterised by a progressive increase of their carbon content. In ordinary cultivated soils these changes cannot be traced owing to the constant addition of fresh organic matter, and cultural operations, but in peat beds, where the deposits remain undisturbed for many years, the stages are indicated by the increasing carbon content of the humus from varying depths. This was shown by Detmer [1871], who gives the following figures:

	C	H	O
Light brown peat, surface	52·14	7·03	40·19
Brown peat, 1 foot	57·75	5·43	36·02
Dark peat, 7 feet	62·02	5·21	30·67
Black peat, 14 feet	64·07	5·01	26·87

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Thus in peat bogs the carbohydrates of decaying organic matter may possibly pass through changes very similar to those observed when sugar is heated to a high temperature: sugar, caramelan, humic acid, humin, and finally carbonisation (peat coal). The fact established by Van Bemmelen [1900] that humic acid is a colloid body which has the property of uniting with certain radicles explains the various empirical formulae ascribed to humic acid, and the complex nature of humus in general. But underlying this complexity there is the possibility of finding in the series of sugar changes described above two fairly definite groups of substances which serve as a basis for humus formation, the humic group gradually merging into the humin group.

Summary.

1. Sugars on boiling with hydrochloric acid yield a mixture of humic acid and humin bodies, varying in proportion with the different sugars used.

2. The composition of "natural" humic acid from soil or peat, after purification by alcohol, approximates very closely to that of "artificial" (sugar) humic acid.

3. Humic acid and humin are also produced from sugars by the action of various organic acids: lactic, acetic, propionic, butyric, etc.

4. The action of heat alone on sugars produces humic acid and humin bodies, these substances being stages in the process of carbonisation.

5. Humic bodies are not obtainable from proteins free from carbohydrates.

6. The two groups of humic bodies—humic acid and humin—obtained artificially from carbohydrates, indicate a basis for the natural processes of humus formation.

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XXIV. THE FREE AMINO NITROGEN OF THE DIFFERENT PROTEINS OF OX AND HORSE SERUM.

By PERCIVAL HARTLEY.

From the Biochemical Department, Lister Institute of Preventive Medicine.

(*Received May 11th, 1915.*)

In a recent communication van Slyke and Birchard [1913] have shown that when certain native proteins are treated with nitrous acid the amount of nitrogen formed as a result of the reaction is equal to one-half the lysine nitrogen of the protein. They suggest that one of the amino groups of lysine, the ω-group, exists free in the intact protein molecule, and, moreover, that this ω-amino nitrogen of the lysine represents practically the entire amount of free amino nitrogen which can be demonstrated by the nitrous acid method of van Slyke. All the amino acids, except lysine, react quantitatively with nitrous acid in three to four minutes; lysine reacts more slowly, thirty minutes being required for complete reaction, and van Slyke and Birchard show that reaction between nitrous acid and the native proteins also requires about thirty minutes to attain completion. The authors quote observations of other workers which support their view that the free amino nitrogen of native proteins is due to the ω-amino group of the lysine which they contain. Gelatin, caseinogen and serum globulin after treatment with nitrous acid yield no lysine on subsequent hydrolysis with acid. Clupeine, which contains no lysine, yields no nitrogen on treatment with nitrous acid, while sturine and cyprinine, which contain lysine, do. It has also been shown that certain protamines and proteins which contain lysine reveal free amino groups by the formal titration method of Sörensen, while those which contain no lysine do not.

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The analysis of the different proteins of ox and horse serum, an account of which has recently appeared [Hartley, 1914], shows that albumin differs in several important respects from the globulins. In particular it has been found that albumin contains a much larger amount of lysine than the globulins. The experiments described in this paper have been carried out in order to study the relationship between the free amino nitrogen of the different serum proteins and their lysine content.

The preparation of the different serum proteins has been described in the earlier paper. For the determination of the amino nitrogen the following procedure was adopted in each case. Two weighed quantities of the air-dried preparations were taken: one of these was used for the determination of the total nitrogen of the protein by Kjeldahl's method and the other was dissolved in water or very dilute alkali in a standard 15 cc. flask. Ten cc. of this solution were treated with nitrous acid in van Slyke's apparatus and the free amino nitrogen of the protein determined. The reaction was conducted in the standard size apparatus described by van Slyke [1912], 1 cc. of octyl alcohol being added in each case to prevent frothing. The mixture of nitrous acid and protein was shaken at intervals for twenty-five minutes and finally shaken continuously for five minutes.

The results of the determination of the total nitrogen and the free amino nitrogen are collected in the following tables.

Total globulin is the name given to the proteins precipitated from the diluted serum by half saturation with ammonium sulphate. It consists of pseudoglobulin and euglobulin. Euglobulin *A* was prepared from the total globulin by salting out with ammonium sulphate, and euglobulin *P* was prepared by the dilution and acidification method of Panum.

TABLE I.

Total Nitrogen Determinations.

No.	Protein	Quantity taken g.	N/10 H_2SO_4 neutralised cc.	Nitrogen mgm.
1.	Albumin (ox)	0·2065	18·8	26·32
2.	Total globulin (ox)	0·2110	19·9	27·86
3.	Albumin (horse)	0·2562	23·7	33·18
4.	Total globulin (horse)	0·2604	24·9	34·86
5.	Pseudoglobulin (horse) ...	0·2882	27·1	37·94
6.	Euglobulin *A* (horse)	0·2894	26·4	36·96
7.	Euglobulin *P* (horse)	0·3047	25·2	35·28
8.	Whole proteins (horse) ...	0·2761	23·3	32·62

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TABLE II.

Amino Nitrogen Determinations.

No.	Protein	Quantity taken gm.	N gas cc.	Temp. °C.	Pressure mm.	Amino N mgm.	Per cent. of total N as free NH_2
1.	Albumin (ox)	0·5157	9·9	16	755	5·70	8·7
2.	Total globulin (ox) ..	0·5013	4·8	16	758	2·80	4·2
3.	Albumin (horse)	0·3875	7·0	17	762	4·05	8·0
4.	Total globulin (horse)	0·3932	5·2	19	762	2·98	5·6
5.	Pseudoglobulin (horse)	0·2806	3·1	15	760	1·81	4·6
6.	Euglobulin *A* (horse)	0·4096	5·4	17	749	3·07	5·8
7.	Euglobulin *P* (horse)	0·4050	4·3	17	749	2·44	5·2
8.	Whole protein (horse)	0·3963	5·1	19	762	2·92	6·2

In Table III the results of the determination of the free amino nitrogen are given and compared with the lysine content of the different proteins. The figures for the lysine are taken from the earlier paper [1914] dealing with the analysis of the different proteins of ox and horse serum.

TABLE III.

No.	Protein	Per cent. of total nitrogen as Free amino N	Half lysine N
1.	Albumin (ox)	8·7	8·15
2.	Total globulin (ox)	4·2	4·50
3.	Albumin (horse)	8·0	7·95
4.	Total globulin (horse) ...	5·6	5·40
5.	Pseudoglobulin (horse) ...	4·6	4·90
6.	Euglobulin *A* (horse)	5·8	4·97
7.	Euglobulin *P* (horse)	5·2	5·05
8.	Whole proteins (horse) ..	6·2	6·40

Except in the case of Euglobulin *A* (horse) in which the result is a little high, the free amino nitrogen of the proteins of ox and horse serum is equal to one-half the lysine nitrogen. The results are in agreement with those obtained by van Slyke and Birchard for other native proteins and lend support to the suggestion advanced by them that in the intact protein molecule the ω-amino group of the lysine exists free, and, further, that this group represents practically the whole of the free amino nitrogen determinable by means of nitrous acid.

The results given above, taken in conjunction with those of van Slyke and Birchard, indicate that the lysine content of a native protein may be rapidly estimated by determining the amino nitrogen and the total nitrogen of the protein.

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XXV. SOIL ORGANIC MATTER AS A CULTURE MEDIUM FOR *AZOTOBACTER*.

By FLORENCE ANNIE MOCKERIDGE.

From the Botanical Laboratories, King's College, London.

(*Received May 10th, 1915.*)

Not the least important of the functions performed by soil organic matter is that of providing food material for the bacterial flora upon the activity of which the fertility of the soil so largely depends. The problem of maintaining soil fertility often resolves itself into a question of promoting bacterial activity, and it is safe to say that the most important of all the soil organisms are those concerned in the nitrogen cycle. The nitrifying organisms assimilate the carbon dioxide of the air, and are thus independent of organic carbon; the ammonifying organisms can do their work when provided with the humus, or any organic matter containing nitrogen, so long as potassium salts are present [Dumont, 1905]; but the nitrogen fixing organisms, and especially *Azotobacter*, are generally supposed to be somewhat sensitive in their requirements, flourishing only upon specialised non-nitrogenous organic compounds. A wide variety of such substances pass into the soil from the natural accumulations of humus by the ploughing in of green crops and in the application of rotted farm and stable manure. These compounds undergo decomposition in the soil through the agency of many micro-organisms, including the spores and mycelia of the higher fungi [C. van Iterson, 1904]. The various enzymes which are introduced with the organic matter remain active in the soil [Woods, 1899], and assist in the further decomposition of these compounds; while the intact roots of plants have the power of oxidising the organic matter and bringing about an appreciable change in its composition [Molisch, 1887; Schreiner and Reed, 1909]. The higher organisms

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such as worms are active in splitting up the fats and other complex substances; while in addition to these organic agencies at work, there must be taken into account the purely chemical changes which are constantly taking place. The nett result of all these reactions is the production of a large number of organic compounds, both nitrogenous and non-nitrogenous; the latter ranging in complexity from carbohydrates down to the simplest alcohols. It is generally assumed that carbohydrate in some form is essential for the nutrition of *Azotobacter*, although cultures have been obtained upon certain other substances, notably some of the vegetable acids, and the object of the present investigation was to discover how far the organism is capable of utilising the wide range of non-nitrogenous organic compounds which occur in the soil.

Method of work.

A uniform method of procedure was adopted throughout the work. The culture medium employed, with variation of the source of organic carbon only, was that used by Bottomley and given in detail in a previous communication by the writer [Mockeridge, 1912]. For the determination of the nitrogen fixing power of the organism upon each substance, 50 cc. portions of the medium, containing 1 % of the nutrient, were placed in each of six 300 cc. Erlenmeyer flasks, and sterilised in the autoclave at a temperature of 140°. Where organic acids were employed, sufficient calcium carbonate to neutralise them was added to each flask, and in the case of volatile organic substances, these were not added to the medium until the flasks containing the inorganic salts in solution had cooled down after sterilisation. Each flask was provided with a sand slope consisting of 50 grams of pure sterile sand, and was then inoculated with 1 cc. of a uniform suspension in distilled water of *Azotobacter* from a mannitol-agar plate. Two flasks of each series were then again sterilised to serve as controls, and the whole series was incubated at a temperature of 26° until all trace of the organic substance originally supplied had disappeared from the medium, when the contents of each flask were analysed by the Kjeldahl process for its nitrogen content.

Humates.

It is doubtful whether soluble humates can serve as a source of food material for *Azotobacter*, although it has been proved [Krzemieniewski, 1908] that they stimulate the activity of the organism, when the latter is already

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provided with another nutrient; and if this takes place in artificial media and under the abnormal conditions of the laboratory tests, the probability is that it occurs more markedly still in the soil. In the present work it was found possible to obtain a growth of *Azotobacter* upon a solution of ammonium humate containing the requisite mineral salts, although no increase in the nitrogen content of the medium took place; but such growth could not be obtained upon media containing potassium or calcium humate without any other source of organic carbon. The organism thus appears to be able to utilise the humates as a source of energy if provided with nitrogen already fixed, and ammonium humate is present in all fertile soils in small, though appreciable, quantities; the ammonia produced by the decomposition and ammonifying organisms uniting with the humic acid in the organic matter.

Polysaccharides.

The widespread occurrence of various gums and starches in vegetable tissues is well known, and these substances must be transferred to the soil in all accumulations of plant débris. The value of dextrins as a source of carbon for mixed cultures of nitrogen-fixing organisms has already been pointed out [Bottomley, 1912]; and since the enzyme (amylase) directly concerned in their production is practically always found in foliage leaves [Brown and Morris, 1893], the addition of these bodies to the soil in green manures, as well as their production in the soil itself and in rotting manure-heaps, by hydrolysis of cellulose and starch are matters of certainty. Inulin is also a common food reserve in plants. As therefore the various polysaccharides must naturally occur in soils, an examination was made of the availability of certain of them as nutrients for *Azotobacter*. Gum arabic, gum tragacanth, starch, dextrin and inulin were tested, and all proved to be readily available for the organism.

The fixations obtained were as follows:

TABLE I.

Substance	Nitrogen fixed on 1 gram (average of 4 determinations). Mg.	Time taken completely to use 1 gram of the substance. Days
Gum arabic	6·13	60
Gum tragacanth .	9·13	20
Rice starch	6·40	18
Potato starch ...	5·93	20
Dextrin	6·62	20
Inulin	9·76	16

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THE RESPIRATORY FUNCTION OF THE BLOOD

BY

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Extract from the Preface

At one time, which seems too long ago, most of my leisure was spent in boats. In them I learned what little I know of research, not of technique or of physiology, but of the qualities essential to those who would venture beyond the visible horizon.

The story of my physiological "ventures" will be found in the following pages. Sometimes I have sailed single handed, sometimes I have been one of a crew, sometimes I have sent the ship's boat on some expedition without me. Any merit which attaches to my narrative lies in the fact that it is in some sense at first hand.

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The Respiratory Function of the Blood

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some feathers for the whipping, and a small stoppered test tube in which to put the whipped blood. At the top of the ascent I obtained a sample of alveolar air which proved to have a partial pressure of 35 mm. of CO_2 as opposed to 40 mm. at the sea level, also a sample of my blood for analysis. The analysis was two-fold—firstly the blood was exposed to 17 mm. oxygen pressure in the absence of CO_2 for the purpose of determining by Mathison's method* the degree of acidosis, if any. The blood at 17 mm. was, as the result of two determinations, 54 °/₀ and 57 °/₀ saturated with oxygen, whilst before the climb it was

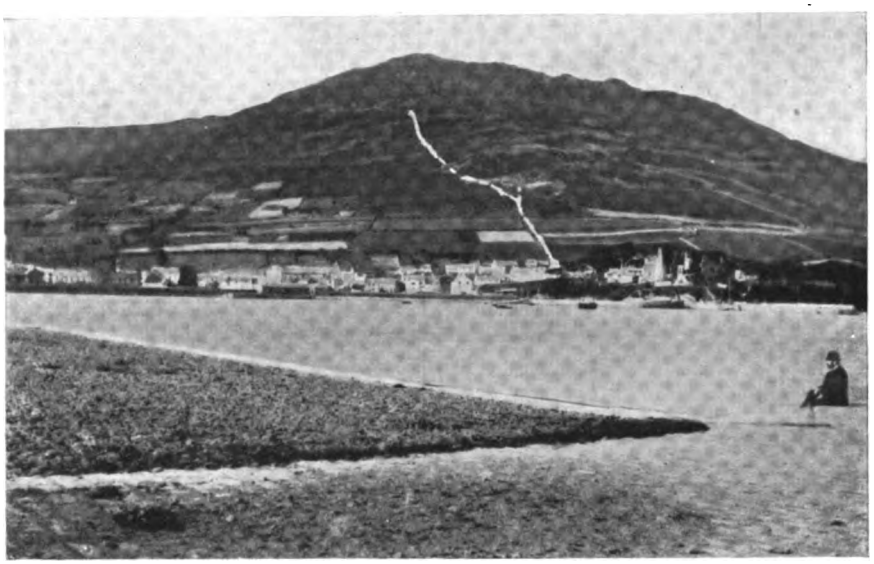

Fig. 112.—Carlingford, showing the climb of 1000 feet.

75 °/₀. The difference corresponds to an added amount of acid which is equivalent to ·023 °/₀ lactic acid.

Against this however the amount of CO_2 in the alveolar air and presumably in arterial blood went down from 40 mm. to 35 mm. The question at once arose, did this fall in CO_2 compensate for the increase in other acids? In other words, was the actual blood in the body pleonectic, mesectic, or meionectic? The answer of course could only be found out by experiment. This experiment formed the second part of the analysis. The blood taken at the end of the ascent was exposed to oxygen pressures in the presence of CO_2

* See p. 257.

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higher altitudes at which the working life of man is carried out on a normal scale. As a station for the study of climate the Cañadas offer, among other advantages, a relative immunity from wind. This in view of the investigations published by Lyth [1] is a factor worthy of consideration. The island of Teneriffe consists roughly of a huge crater about 8000—9000 feet in height. The diameter from lip to

FIG. 116.—The Cañadas showing the living house and the laboratory. Espigone, one of the summits, on the lip of the "old crater" in the background. (Douglas.)

lip is eight miles. On the south side of the island the lip is incomplete. The inside of the tip is a steep, not quite precipitous, cliff, down which you must climb for a thousand or two feet, unless you enter the crater as we did by a gap in the cliffs we called the Portillio. We were then inside the old crater; our back was to the cliff, which in places rises in named summits, Guajara and Espigone for instance. Our faces were towards a level plateau of sand.

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Sugars.

Sugar in some form is the usual nutrient supplied to *Azotobacter* in artificial cultures, and the power of the organism to utilise most of the more readily obtainable sugars was tested. These included pentoses and hexoses among the monosaccharides, and some of the disaccharides.

The results obtained were as follows:

TABLE II.

Sugar	Nitrogen fixation on 1 gram (average of 4 determinations). Mg.	Time taken completely to use 1 gram. Days
Arabinose	9·28	30
Xylose	9·00	28
Dextrose	6·57	20
Laevulose.....	10·32	18
Galactose	6·20	22
Sucrose	7·28	22
Maltose	7·55	17
Lactose	3·39	34

The fact that the organism is capable of utilising two of the pentoses, arabinose and xylose, renders it probable that other members of the group, which occur somewhat widely in nature, are also available; for pentose sugars are formed both in the soil and in the manure-heap by the hydrolysing action of certain bacteria and enzymes upon gums, pectin compounds, naturally occurring glucosides, nucleic acids, etc. Recently a pentosan of undetermined nature, and also rhamnose itself, have been isolated from samples of loam [Schreiner and Shorey, 1910; Shorey, 1913]. Similarly the wide distribution of the hexoses and disaccharides in nature, and the variety of the agencies by which they may be produced in all decomposing vegetable matter, indicate a wide range of available food for *Azotobacter* in the soil.

Alcohols.

Among the substances tested as a source of food for *Azotobacter* were some of the monohydric alcohols, besides representatives of some of the more complex alcoholic compounds, as ethylene glycol (a dihydric alcohol), glycerol (trihydric), erythritol (tetrahydric) and mannitol (hexahydric). The fact that substances of an alcoholic nature are readily assimilable by *Azotobacter* is shown in the following table:

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TABLE III.

Alcohol	Nitrogen fixed on 1 gram (average of 4 determinations). Mg.	Time taken completely to use 1 gram. Days
Methyl alcohol ..	2·1	42
Ethyl alcohol ...	4·02	34
Propyl alcohol ..	9·2	22
Isobutyl alcohol .	4·69	72
Ethylene glycol .	16·74	34
Glycerol	5·0	56
Erythritol	4·88	60
Mannitol	11·62	15

Only one determination of the nitrogen fixed upon ethylene glycol was made, on account of the difficulty of obtaining larger quantities of the compound; but a very copious growth of the organism upon this nutrient was obtained.

Flasks containing amyl alcohol and inoculated with *Azotobacter* showed no growth of the organism at the end of a month, but upon a solid medium obtained by the use of agar-agar a fair growth appeared upon this nutrient. No determinations were made of the nitrogen fixed.

The alcohols are of very frequent occurrence in nature, and appear to be sufficiently abundant in soil organic matter to warrant their inclusion among the bacterial foodstuffs of the humus. The possible sources in the soil of the alcohols mentioned in the above table are indicated below. Methyl alcohol occurs in combination in various plants, and is produced in the free state in cow dung by the action of *B. boöcopricus*. Ethyl alcohol is a well-known product of yeast fermentations and of practically all bacterial action, while propyl alcohol usually accompanies it in small quantities as a by-product. Butyl alcohol is formed during butyric fermentation, while under certain conditions *B. amylozyma* will produce amyl alcohol. Ethylene glycol is very probably a by-product of bacterial action in the soil, for one of its higher homologues, butylene glycol, has been shown to be formed during the action of *B. lactis aerogenes* on glucose and mannitol [Harden and Walpole, 1906]. Glycerol is a well-known by-product in yeast fermentations and in many bacterial actions; erythritol is found in the free state in lichens, mosses and seaweeds, while mannitol occurs naturally in many plants. The last-named substance has been isolated in fairly large quantities from one soil [Shorey, 1913].

Hence it is probable that substances of an alcoholic nature occur fairly widely in the soil, and are sufficiently abundant to provide a convenient source of food for *Azotobacter*.

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Vegetable acids (other than aliphatic).

The organic acids are constant products of decomposition of practically all organic matter, and so probably most of them occur in soils and manures. The nitrogen-fixing power of *Azotobacter* when grown upon a medium containing the calcium salts of the organic acids was tested, with the following results:

TABLE IV.

Acid	Nitrogen fixed upon 1 gram (average of 4 determinations) Mg.	Time taken to use completely 1 gram. Days
Citric	6·44	48
Malic	5·19	16
Tartaric	4·54	28
Racemic ...	2·77	50
Succinic	8·60	20
Malonic	5·32	20
Mucic	6·79	40
Fumaric ...	2·00	50
Maleic	1·88	50
Glycollic ...	1·75	60
Lactic	12·01	17

The vegetable acids are widely distributed in nature, citric acid, malic acid and tartaric acid and its stereo-isomers being among the most commonly occurring; the first-named, besides its occurrence in the free state, arises also as a product of the action of various micro-organisms. Succinic acid is also found in many plants, besides which it is a product of putrefaction and occurs in the urine of goats, horses, rabbits and cows, so that it is probably transferred to the soil in rotting manure, in which is also to be found lactic acid—a product of the lactic fermentation of carbohydrates in the presence of nitrogenous animal matter. The remaining acids tested, mucic, glycollic, maleic, fumaric and malonic, though they do not occur in plant residues, so far as is known at present, may readily be produced in soil or manure from the other acids enumerated, or from other organic substances by the various agencies at work during the decomposition of soil organic matter. That mucic acid, for instance, probably exists in the soil is indicated by the fact that saccharic acid, an isomeric form, has actually been isolated [Shorey, 1913], although this is not known to occur in plants. From the same soil were isolated oxalic, succinic and acrylic acids, while from other samples paraffinic, lignoceric, α-hydroxystearic and some resin acids have been isolated [Schreiner and Shorey, 1910], while still other soils have been found to contain dihydroxystearic, picolinecarboxylic and agroceric acids [Schreiner and Shorey, 1909].

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Meconic acid was found to be useless as food for *Azotobacter*, but the substance itself is not inhibitory to the organism, for good cultures were obtained upon mannitol-agar plates containing meconic acid. Probably some of its products of decomposition have a deleterious effect. The poisonous nature of oxalic acid accounts for the fact that it is not available as a source of food for the organism.

Organic acids of any kind are useless to *Azotobacter* until neutralised by lime or some other base, but this presents no difficulty, since there is abundance of lime for the purpose in all fertile soils.

Fatty acids.

In the series of aliphatic acids, those tested with regard to their availability for *Azotobacter* included formic, acetic, propionic, butyric, isovaleric, stearic and palmitic acids. On the last three no growth was obtained in liquid culture, but when the medium was solidified by the addition of 2 % agar-agar, and poured into Petri dishes, a fairly abundant growth was obtained upon the surface. The organism is thus capable of utilising these substances as a source of energy, although it requires special conditions, probably connected with air supply. No determination of the nitrogen fixed upon these substances was made. Formic, acetic, propionic and butyric acids formed readily available sources of food, and the figures obtained upon these media are given in greater detail than has hitherto been the case, since further reference will be made later to these results.

The fatty acids are very widely distributed in nature. The more complex fats and oils are of almost universal occurrence in the vegetable kingdom, being found as reserve stores in fruits and seeds. Oil cakes of various kinds form a large part of the diet of animals at fattening time, so that an appreciable quantity of undigested fat and oil is probably transferred to the manure heap. These complex bodies of a fatty nature undergo decomposition through bacterial action under certain conditions of light and aeration with production of the simpler aliphatic acids. Reserves of fat in plants are always accompanied by the fat splitting enzymes, so that in the soil the decomposition of the fat into glycerol and a fatty acid, most commonly oleic, palmitic and stearic, may proceed. The lower aliphatic acids are also distributed in fair quantity throughout rotting manures, being formed by the action of bacteria upon many substances, especially upon the degradation products of the proteins; so that members of this group, when

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neutralised by lime or some other base, serve as a readily available source of food for *Azotobacter*.

TABLE V.

Nature of medium	Nitrogen content, mg.	Fixation on ½ g., mg.	Fixation on 1 g., mg.	Mean Fixation, mg.	Time taken to use 1 g.
50 cc. 1 % formic acid control	0·29				
	0·29				
,, ,, with *Azotobacter*	0·91	0·62	1·24	1·47	60 days
,, ,, ,,	1·22	0·93	1·86		
,, ,, ,,	1·06	0·77	1·54		
,, ,, ,,	0·91	0·62	1·24		
50 cc. 1 % acetic acid control	0·16				
	0·16				
,, ,, with *Azotobacter*	2·26	2·10	4·20	3·77	24 days
,, ,, ,,	2·12	1·96	3·92		
,, ,, ,,	1·98	1·82	3·64		
,, ,, ,,	1·83	1·67	3·34		
50 cc. 1 % propionic acid control	0·41				
	0·41				
,, ,, with *Azotobacter*	3·14	2·72	5·44	5·16	26 days
,, ,, ,,	2·86	2·44	4·88		
,, ,, ,,	3·00	2·58	5·16		
,, ,, ,,	3·00	2·58	5·16		
50 cc. 1 % butyric acid control	0·14				
	0·14				
,, ,, with *Azotobacter*	3·18	3·04	6·08	6·08	48 days
,, ,, ,,	3·04	2·90	5·80		
,, ,, ,,	3·32	3·18	6·36		
,, ,, ,,	3·19	3·05	6·10		

Esters.

The ethereal salts, or esters, occur widely, although in small quantity, in the essences of flowers and fruits and certain leaves. They probably find their way into the soil from these sources, and there undergo decomposition, through the agency of the lipases or other hydrolysing agents, into their two primary constituents; but even in the free state they are able to serve as a source of energy for *Azotobacter*. Two only of the simple esters were tested: ethyl and butyl acetates, and upon neither of these in liquid culture could any growth be obtained. Upon agar plates containing these substances, however, a fairly abundant, though slow, growth took place.

Glucosides.

The glucosides are of very frequent occurrence in plant substances, but their availability as food for this nitrogen-fixing organism appears to be restricted somewhat by the products of their decomposition. An abundant

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growth, but a very small fixation of nitrogen, was obtained upon a solution of amygdalin, a glucoside occurring in bitter almonds and the kernels of several stone fruits. This glucoside upon hydrolysis gives rise to glucose, benzaldehyde and hydrocyanic acid, and these are the products of the action of yeast upon it. The glucose, of course, is immediately available for *Azotobacter*, and neither the hydrocyanic acid nor the benzaldehyde appears to be inhibitory to its growth. Hydrocyanic acid is found extensively in the free state in plants, so that small quantities of this substance are evidently not deleterious to plant life.

Most of the naturally occurring glucosides, e.g. salicin, give an alcohol upon hydrolysis, and salicin was found to be useless for the growth of *Azotobacter*, either in liquid or solid media. This fact points to the conclusion either that the organism has no action on the glucoside or that its first action is a hydrolytic one; for salicin, upon hydrolysis, gives glucose and salicyl alcohol, which latter is a powerful antiseptic, and would accordingly inhibit the growth of the organism. The substance itself is not harmful to *Azotobacter*, for an abundant growth of the organism was obtained on mannitol-agar plates which contained salicin; a fact which indicates that it must be the decomposition products of the salicin which have a deleterious effect.

Benzene derivatives.

A similar state of affairs was found to obtain with all the benzene derivatives tested. These included phenylacetic acid, mandelic (phenylglycollic) acid, benzyl alcohol, pyrogallol, quinol and catechol. These substances will not serve as a food supply for the organism in either liquid or solid media, yet when mixed into mannitol-agar plates do not inhibit its growth. Such substances as benzyl alcohol, catechol, pyrogallol and quinol are not readily split up chemically, and the great stability of the benzene ring may account for the unsuitability of these substances as food for *Azotobacter*; moreover, from such substances as phenylacetic and phenylglycollic acids there is always the possibility of the formation of phenol, and this product might prevent any further bacterial growth. The inability of the organism to utilise substances of this nature thus apparently depends not upon any harmful effect produced by the compounds themselves, but either upon the injurious secondary products formed by their decomposition, or on the difficulty of breaking up the stable benzene ring.

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General considerations.

The results obtained indicate the wide range of substances which are available as a source of energy for this nitrogen-fixing organism in the soil: substances which occur naturally and may be added to the soil in green manures or in accumulations of vegetable débris; compounds which may be produced by fermentation of rotting manures and added to the soil in that way; and products which arise by secondary and often purely chemical reactions in the soil itself. It is of interest to note also, that the large number of organic substances present in the soil, which the organism cannot utilise, do not necessarily inhibit its growth, so long as some available food material is present; for *Azotobacter* appears to be possessed of considerable selective power.

All classes of compounds do not give equally good returns of nitrogen fixed for the expenditure of food material, and, generally speaking, it seems to be the rule that the longer the time taken to use completely unit mass of the nutrient, the less the nitrogen fixed upon that medium. Though they serve as a general guide, too much reliance must not be placed upon the figures obtained in this respect, for, as the author has pointed out in previous work, the nitrogen-fixing power of the organism varies considerably with the length of time during which it has been grown upon artificial media.

In order to compare correctly the relative availability of a large number of nutrient substances, the same strain of *Azotobacter* should be used throughout for inoculating purposes, and this means that all the cultures required should be incubated at once, whereas in practice the work has to be carried out in sections.

Generally speaking, the carbohydrates give the greatest yield of nitrogen fixed per unit of material consumed, and it is only to be expected that in the breaking down of the more complex molecule more energy should be obtained by the organism, and thus that more growth and consequent fixation of nitrogen should take place. *Azotobacter* is essentially an aerobic organism, and Beijerinck and van Delden [1902] observed that it absorbed oxygen and eliminated carbon dioxide, their observations being confirmed by the careful work of Krzemieniewski [1908]. This points to a slow combustion of the organic material provided; and some idea of the energy thus obtained by the organism can be gained by a calculation of the heat of combustion of the carbon compound supplied. When these figures are calculated for the aliphatic acids and compared with the amounts of nitrogen fixed upon those substances as given in Table V, the following results are obtained:

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TABLE VI.

Acid	Molecular weight	Heat of combust. of 1 gram-molecule	Heat of combust. of 1 gr.	N fixed on 1 gr.	Ratio N fixed : Heat of combust.
Formic	46	68300 cals.	1484 cals.	1·47 mg.	1 : 1009
Acetic	60	230900	3848	3·77	1 : 1020
Propionic ...	74	393500	5317	5·15	1 : 1032
Butyric	88	556100	6319	6·08	1 : 1039

Within the range of experimental error, these ratios may be taken to be approximately constant, a result which points to the conclusion that in these four media, the decomposition of the respective organic constituents follows the same course, a very definite proportion of the energy thus obtained being devoted to the fixation of nitrogen.

It is obvious that, on account of the varied nature of the possible products of decomposition, such comparisons can only be made between substances of similar constitution; but the figures obtained for some of the carbohydrates are of interest.

TABLE VII.

Carbohydrate	Heat of combustion of 1 gram	Fixation on 1 gram	Ratio N fixed. Heat of combustion
Starch ...	4145 cals.	6·40 mg.	1 : 647
Dextrin ..	4145	6·62	1 : 626
Gum arabic	4145	6·13	1 : 676
Inulin	4145	9·76	1 : 424

Allowing for error of experiment, which is considerably greater here than in the case of the simpler compounds, owing to the difficulty of ascertaining that all the secondary products of the carbohydrate have completely disappeared, the ratios for starch, dextrin and gum arabic may be taken to be approximately equal. Assuming that hydrolysis takes place, as it must do, the starch will be converted finally into dextrose, with dextrin and maltose as intermediate products. A comparison of the ratios obtained with these substances is found to be

Starch →	Dextrin →	Maltose →	Dextrose
1 : 647	1 : 626	1 : 598	1 : 585

The ascending order of these ratios bears out the assumption that a hydrolysis of the polyoses takes place to form the simple hexoses, which latter are then oxidised. The ratio obtained for inulin (1 : 424) is surprisingly high as compared with those for starch, dextrin and gum arabic. However, inulin upon hydrolysis gives laevulose, and the ratio between the nitrogen fixed on one gram of laevulose and the heat of combustion of one gram of the same body is found to be 1 : 372, which compares very well with the inulin ratio.

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The close relation which seems to exist in the series of aliphatic acids between the nitrogen fixed and the nutrient consumed is not apparent in all classes of homologous compounds, for the series of monohydric alcohols shows no such relation; and some of the simpler compounds, as ethylene glycol, show a surprisingly high rate of fixation of nitrogen for the heat-energy obtainable. However, nothing is known as to the actual mechanism of fixation. It may be that the nitrogen is attached to very simple organic compounds produced by the splitting up of the nutrient, and that certain substances give decomposition products which are peculiarly adapted to the purpose, while the secondary products from others may be practically useless. On the other hand, a synthesis may take place between the simpler bodies to form substances more complex, before the nitrogen is attached, certain nutrients furnishing the necessary synthetic products with less waste of energy and material than others. That synthesis of non-nitrogenous substances takes place in the cells of *Azotobacter*, apart from nitrogen assimilation, is shown by the fact that appreciable quantities of fat have been found stored up in the cells [Lipman, 1904]. By no means the whole of the energy obtainable from the nutrient is utilised in nitrogen fixation, for the author above quoted states that according to thermo-chemical calculations, one gram of mannitol would allow of the fixation of 97·3 mg. of nitrogen. However, the few data obtained appear sufficient to warrant the conclusion that a detailed study of energy considerations may help to throw some light upon the manner in which the nitrogen is fixed; but no matter what course this may follow, it is evident that any ordinary fertile soil contains abundant food material for the growth of *Azotobacter*.

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XXVI. NOTES ON COLLODION MEMBRANES FOR ULTRAFILTRATION AND PRESSURE DIALYSIS.

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(*Received May 21st, 1915.*)

The differential porosity of gel-membranes to molecules of different sizes may be utilised in a number of different ways all of which are essentially filtration. If the "filtrate" side of the membrane is bathed in any solution other than filtrate the process may be called "dialysis." Filtration under pressure is a very common procedure: but the many advantages to be obtained by dialysis under pressure have not received the study and recognition they undoubtedly deserve.

The difference between ultrafiltration and pressure dialysis.

Pressure dialysis brings about the complete removal of any constituent of the material handled whose molecules are by reason of their size and nature capable of traversing the membrane. Ultrafiltration, on the other hand, permits only the removal of those small molecules which can actually be brought into contact with the filter and pushed through. Hence, in order to separate a mixture of materials into those constituents whose molecules pass through a certain diaphragm and those whose molecules do not, either ultrafiltration or pressure dialysis may be employed. The latter process may be regarded as that which gives a quantitative separation, with the disadvantage that the materials passing through the membrane are recovered in a dilute condition, although the volume of residual solution is completely under control.

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Collodion membranes. The use of membranes of collodion for the processes referred to will always be associated with the names of Rodewald and Kattein [1900], Malfitano [1904], Duclaux [1905, 1907], Moore and Roaf [1907, 1908], Lillie [1907] and Bechold [1907, 1, 2, 3; 1908]. The last author used pressure and introduced the use of formalised gelatin as a filtering medium. To him, too, we are indebted for the word "ultrafiltration," which has now passed into general use. In this matter C. J. Martin [1896] anticipated many later developments by fully describing and illustrating special apparatus by which solutions to be investigated were forced under pressure through films of gelatin or silicic acid. The material impregnated was a Pasteur-Chamberland filter candle, which served as a support against disruption. In conjunction with T. Cherry [1898] such filtrations were used to demonstrate that diphtheria toxin-antitoxin mixtures after two hours' standing at 30° gave toxin-free filtrates, although toxin when alone passed through the filters, and antitoxin, as shown by Brodie [1897] using Martin's method, did not.

My object in this paper is to draw attention to the possibility of standardising accurately a series of filters, suitable for "ultrafiltration" and pressure dialysis operations, which are already in use. Also, to call attention to certain members of the series, that is to say those containing 5 mg. of nitrocotton per sq. cm. and weighing 17·5 mg. per sq. cm. because of contained water. These have been discovered to be impermeable to all antigens tried, although they allow water, salts and simpler molecules to pass freely—a valuable conjunction of properties not shared, as far as I can discover, by the ultrafilters in general use.

To make the matter clear, it may be stated as a result of direct comparative experiments with parchment and from experience in the past with gelatin filters, that attempts to force diphtheria toxin under pressure of two atmospheres through parchment or gelatin would not result in nearly so much filtrate as if one of the "antigen-proof" collodion films to which I have referred were used. Further, as may be seen by referring to the original papers from the references given, they would not be found to keep back antigen to any great extent.

The reason of this must, of course, in the present state of our knowledge of gel structure and the phenomena of filtration generally, be given with reservation. It would appear, however, not an unreasonable suggestion that these films possess an enhanced uniformity of structure and a great porosity. In other words, the channels leading from one side of the filter

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to the other are none of them sufficiently large to let the antigen pass, but there are many of them. On the other hand, it is suggested that in parchment, for example, these holes are of irregular size. Some are large enough to let diphtheria toxin through, but the total amount of water they will allow to pass is, in virtue of the lower general porosity of the structure, less in this case [Glenny and Walpole, 1915].

Collodion used. As a stock solution a 14 per cent. solution by weight of Schering's celloidin in equal parts of alcohol and ether was originally employed. Later the British Xylonite Company have kindly supplied me with a similar material ready dissolved and filtered clear. It is named for reference F 4241 A and supplied by them in tins with convenient screwed stoppers at a price which represents a considerable saving in expense. The solutions when used in the same manner give very similar results as may be judged from the comparative experiments given below (page 287).

The preparation of collodion bags. These have been often described by Malfitano [1904], Bigelow [1907] and many other authors. That they may be made with differences of porosity is generally known. For information about them and instructions how to make them I am indebted to Prof. S. P. L. Sörensen and Dr Christiansen. They are sometimes made by pouring consecutive layers of the solution on to the outside of a test tube rotated by a suitable mechanism. Probably the bags made in this way are more perfect than those made inside test tubes, but special apparatus is required and, I should judge, considerable experience and skill in order to be sure of producing a bag of just the required impermeability. The following is a description of the bags which I have adopted as "standard," though for anything beyond odd quantitative experiments I prefer to use the flat sheets described in some detail later, for they possess certain advantages over any cylindrical bag however perfect.

A test tube is filled with "stock solution," capped with tin foil and after the twelve or fifteen hours required for the bubbles to rise is inverted and clamped so that the mouth is well above the wide mouthed stock bottle. When the flow of collodion ceases the bottle is removed and stoppered and about ten minutes later the drippings are cut away from the test tube with a sharp knife, care being taken not to pull loose the lower edge of its collodion lining. The test tube must, of course, be cut off sharp and not lipped or opened at the mouth in any way. Drying is allowed to continue until the inside when rubbed gently with the finger does not mark at all—a degree which cannot be described precisely—a process taking about half an hour at

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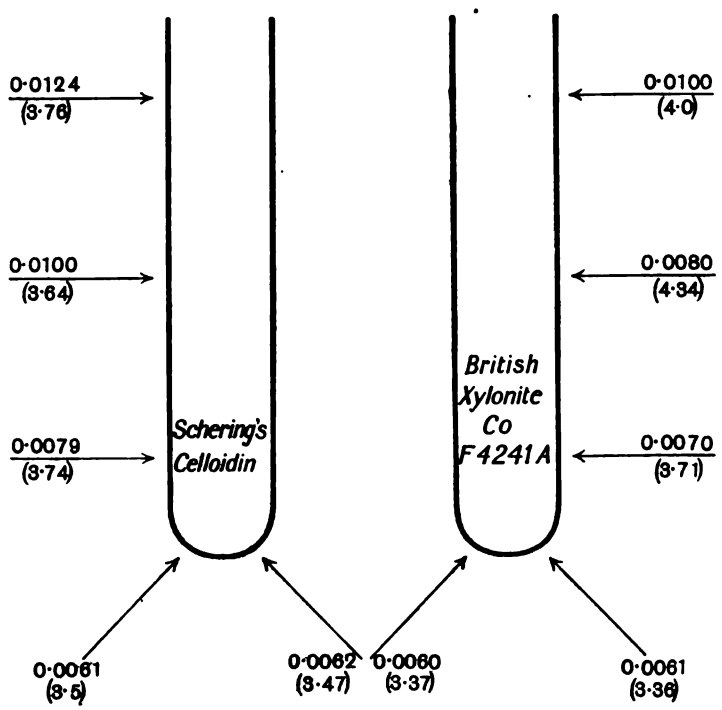
laboratory temperature. The test tube and its invisible collodion lining are now placed under water, and at a later time while still under water the lining may be easily removed and tied to a rubber stopper. It is convenient to remember that these membranes shrink rapidly in air and become less porous, though in cold water they may be stored indefinitely. In hot water they shrink, but may be stretched and moulded without undergoing any apparent permanent change—a property which makes it possible to mount them on rubber stoppers of slightly larger diameter than would otherwise be possible.

Some simple measurements of two bags, made at the same time, one from Schering's celloidin (13·45 per cent. by weight in equal parts dry alcohol and ether) and the other from British Xylonite Company material, F 4241 A (14·66 per cent. in the same solvent), may be of interest. The unavoidable lack of uniformity of bags made in this way is apparent from the diagram below, in which the figures represent the weight per sq. cm. of dry nitrocotton and the "wetness" at the points indicated (see page 292). The latter are in brackets. The dimensions of these bags are 4·5 cm. wide and 22 cm. long.



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Smaller bags than this, even though made with great care, are still less uniform. Three other bags from each material, made at the same time under conditions as similar as it was possible to arrange, were tested. One pair filtered diphtheria toxin, a second pair filtered tetanus toxin, while a third pair were tested by pressure. They both ruptured between two and three atmospheres pressure after stretching considerably.

Ultrafiltration of tetanus toxin. Batch Z. Both bags were fed with toxin at 20 cm. of mercury pressure for about a week; the Schering's celloidin bag passed 2600 cc., while that made from the British Xylonite Company's material passed 2300 cc. The former filtrate gave a very faint indication of toxicity: none could be detected in the latter.

Ultrafiltration of diphtheria toxin (mixture of equal parts J 2133 and J 2159, L_+ dose 0·40 cc.: nitrogen = 5·3 mg. per cc.). The pressure applied equally to both bags was 20 cm. of mercury for twelve days. The bag from Schering's celloidin allowed 1875 cc. to pass; that from British Xylonite Company material filtered 2155 cc. No toxin could be detected in the latter filtrate: 1 cc. of the former gave a slight swelling in the guinea pig.

It may be remarked in passing that the residues in the bags were, at this juncture, surrounded by 0·3 per cent. phenol changed daily for three days and then examined. The volumes were 100, 85 cc.; the nitrogen 2·83, 2·66 mg. per cc.; and the L_+ dose 0·022, 0·018 cc. These figures correspond to concentration of binding units per mg. of nitrogen of 33, 43 times and yields of 81, 89 per cent. respectively.

Apparatus.

The description of various ways in which these bags have been mounted for use in the experiments described in this and in the following paper may be of interest.

The simple mounting of one collodion bag shown in Fig. 1 will be found satisfactory for either filtration or pressure dialysis as long as care is taken to make the apparatus really air-tight, or else to attend to it every few hours. The wide glass tube *b* acts as reservoir for some of the fluid which will ultimately replace that forced through the bag, and also for air under pressure. If an auxiliary air vessel fitted with valve and gauge be attached to *a*, instead of the valve and gauge only, the apparatus can be left unattended for days and maintain the desired pressure. In fact, I have found it convenient to have several such vessels ready fitted for use when required: one of them is shown in Fig. 3 (p. 290). By means of a branched tube it can, of course,

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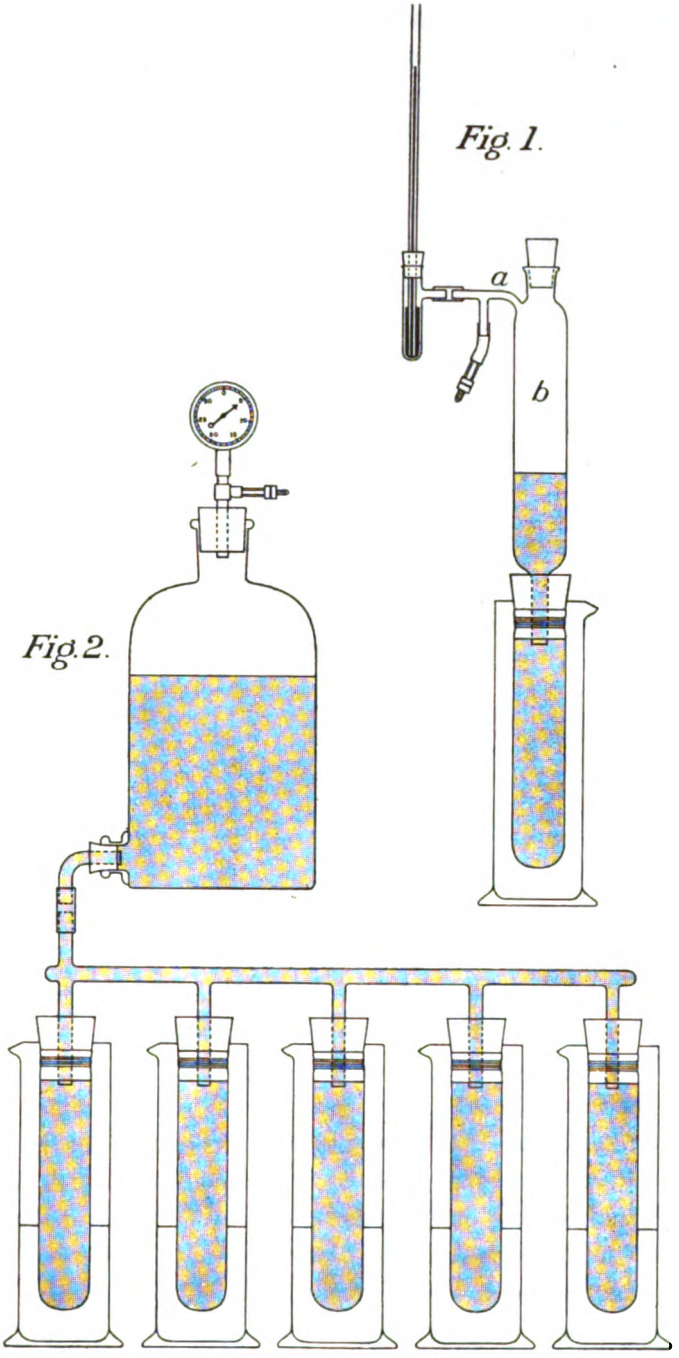

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supply several pieces of apparatus like that sketched in Fig. 1 with air at the same pressure. A convenient mounting for five such bags all holding the same solution is shown in Fig. 2—they are all fed at the same pressure and the filtrates may be compared and so differences in bags investigated. When used for pressure dialysis the water in the jars must be changed from time to time. The inconvenience of not being able to get at the contents of the bags without dismounting the apparatus naturally suggests a continuous apparatus such as that in Fig. 3 for pressure dialysis. The arrangement whereby the toxin and 0·3 per cent. phenol flow in opposite directions effects a very considerable economy in distilled water. The apparatus needs no attention except daily to fill the aspirator with 0·3 per cent. phenol, pump a little air into the reservoir and run off the yield of pressure-dialysed material at *d*. The bottles indicated on the left are double Winchesters holding 5 litres each.

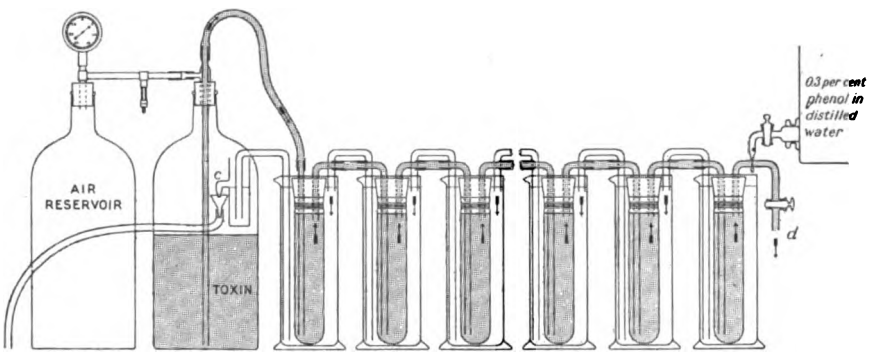

Fig. 3.

The overflow at *c* empties direct to the drains. In two sets of this kind the process has gone on for upwards of three months, in each case without any signs of the bags becoming choked. Apart from the economy of bags, for a set of eight have handled 100 litres of toxin and are still in use, there is possibly the additional advantage that the loss due to adsorption in the bag is minimal, since the volume of toxin handled by each bag is so great. Further details of the working of this apparatus are given in the following paper [Glenny and Walpole, 1915, p. 301].

To avoid the clumsiness of large scale operations in glass apparatus, and the limitation of pressure to that which unsupported bags will stand, it is an obvious step to transfer these operations to properly designed metal

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apparatus in which use is made of flat membranes. These, unlike the collodion bags described, are uniform all over and possess the advantages of being capable of accurate calibration and reproduction.

The preparation of flat collodion sheets. Although in practical concentrations a great deal can be done without the use of flat membranes, for a bag can always be made on the "safe" side with a corresponding loss of rapidity of filtration and other properties of more or less value, nevertheless the latter have many advantages. For large scale technical operations impregnated cloths offer greater permanence and strength, but for research work and technical operations on the smaller scale, where there is opportunity to use a reasonable amount of care with the resulting films, I have taken advantage of the increased simplicity of films of water and nitrocotton only. The absence of supporting material such as cloth or canvas makes it much easier to study the properties of these systems accurately, to reproduce them at will and to arrive at a nice discrimination as to the most suitable membrane for any particular purpose. If a thin membrane of high porosity and therefore low tensile strength be advisable for a particular filtration, I suggest that a fine nickel wire gauze or a cloth to support it should be placed not in the membrane but behind it. The homogeneity of the membrane is then not disturbed and measurements such as thickness, tensile strength, permeability to water, water content, etc., bear results which are more intimately applicable to a rational study of the problem in hand.

A square or round sheet of plate glass larger than the finished filter required is levelled accurately, and on to it is poured a measured quantity of "stock" solution suitably diluted with a mixture of equal parts of dry alcohol and ether. Care should be taken that the collodion solution is poured on to the centre of the glass, and that it is sufficiently diluted to run right to the edge. As it will not run over unless an unnecessarily large volume be taken, the glass really acts as a very shallow dish. As evaporation proceeds, the film slowly hardens until it is decided to transfer the glass plate with its attached film to water, beneath which they are separated after the lapse of a few minutes by one steady pull. At any time later a filter the exact size required is cut out of the film with a template and a knife. A good commercial half-inch plate glass is cheap and quite flat enough to give films, say eight inches across, differing between their thickest and thinnest parts by only 40 μ or less. To obtain such films I have taken pieces of glass 12 inches square. Square films will be found thinner at the corners than at the centres of the sides if the volume of collodion poured be insufficient.

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A tendency which sometimes occurs to curl up and leave the glass in dry or warm weather may be prevented by painting the edges of the hardening film with "Collodion Flexible B.P."

Now the degree and temperature of nitration determine the physical properties of nitrated cotton to a most marked extent, so that the properties of the film ultimately obtained depend upon a host of factors, of varying degrees of importance, starting with the manner of nitration of the cotton and ending with the pressure applied to it in use. To characterise membranes therefore so that they may be reproduced and their individual properties discussed, the only possible plan is to take in every case a quantitative account of the result of all variables in procedure which are recognised and utilised as such, and by using stock solutions either identical or as similar as possible, and constant conditions, to reduce the other factors as far as is experimentally convenient to constancy.

The variables I have utilised are two in number and both of them are readily controlled and expressed numerically. The first is the weight of dry nitrocotton per sq. cm. (m) of the finished film. This is determined beforehand, for the strength and volume of the solution are known as is also the area of the plate over which it is poured. The second is the "wetness" of the resulting film, a property, intimately connected with its porosity, which depends primarily upon the amount of solvent still remaining in the nitrocotton on the glass plate before it is plunged into water. As a numerical expression of the "wetness" (w) of a filter I have found it convenient to use the ratio of its weight to that of the dry nitrocotton it contains.

For example, 157 cc. of a 3·5 times diluted "stock" solution of Schering's celloidin (13·5 per cent. by weight) were poured on to a circular plate 30·5 cm. diameter.

Hence $$m = 0{\cdot}0082 \text{ g.}$$

The weight of a circular filter 23·5 cm. across cut out of this sheet was 17·5 g. The weight of dry nitrocotton it contains is therefore $\frac{0{\cdot}0082 \times \pi \times 23{\cdot}5^2}{4} = 3{\cdot}56$ g.

Hence $$w = \frac{17{\cdot}5}{3{\cdot}56} = 4{\cdot}91.$$

Direct determinations, by weighing, on small pieces from the remainder of the film after the circular filter had been cut out gave

$$m = 0{\cdot}0081 \text{ g.}$$
$$w = 5{\cdot}02.$$

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Now, to test the uniformity of a film it is often convenient to make a few measurements of thickness (t) with a micrometer. The formula connecting m, t and w is found, in practice, to be that which follows at once from the assumption that the thickness of the film is the sum of the thicknesses of the nitrocotton and water in it. The density of nitrocotton may be taken as 2.

$$t = m\left(w - 1 + \frac{1}{\Delta}\right) = m\,(w - 0{\cdot}50).$$

Hence, when making filters to specification it is more convenient not to use the balance at all, but to use a micrometer not only for testing the uniformity of the finished filter, but also for the purpose of determining the correct drying time for any particular film. A thin strip from any part of the film which is just clear of that part which will be required for the finished filter is cut on the glass, peeled off, plunged into water and examined with a micrometer a few minutes later. The thickness gives at once information as to how far the drying has progressed.

Two experimental films, the drying of which was followed in this way, furnished the examples given below:

Example 1. A 13·5 per cent. solution by weight of Schering's celloidin had been diluted 4·5 times. Of this 150 cc. were poured on to a plate 30·5 cm. square, and strips were cut from time to time. Each was immediately after removal from the glass plunged into water, and its thickness measured as the mean of five readings at equidistant points on that part of the strip which would be included in a filter 20·5 cm. square. Direct determinations of m and w were made by weighing pieces of known area both wet and after drying to constant weight. The temperature of the room was 10°–12°.

Example 2. A film containing more nitrocotton per sq. cm. was made by pouring 200 cc. of the "stock" solution above after 3·5 times dilution on to a square plate of the same size. Temperature, 12°–14°.

	Hours of drying before strip was pulled off plate	Mean thickness, t, after immersion in water cm.	Maximum variation from mean thickness μ	m by weighing pieces of known area dried to constant weight	w by weighing pieces wet and after drying to constant weight	w calculated from formula $t = m \times (w - 0{\cdot}5)$
Example 1	2	0·0435	−5	0·00488	9·28	9·41
	3	0·0322	+3	0·00493	6·97	7·03
	4	0·0182	−12	0·00515	4·00	4·03
	5	0·0110	0	0·00503	2·63	2·68
	6	0·0094	¬4	0·00512	2·29	2·33
Example 2	2·25	0·0587	+17	—	7·16	—
	3	0·0484	+16	0·00717	7·29	7·25
	4	0·0337	+13	0·00679	5·48	5·46
	5	0·0194	+13	0·00635	3·56	3·55
	6	0·0117	+3	0·00684	2·15	2·21

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Press for the use of flat sheets. Fortunately these collodion-water membranes make excellent joints, so that the construction of a press (Fig. 4) to take them without leaking is a comparatively easy matter, and as there is no precipitate to be dealt with, the frames may be thin and narrow and the channels small. Instead of horizontal channels leading from frame to frame I have drilled the frames and soldered in nipples over which suitable rubber tube may be passed. Such a connection between any one nipple and another is instantly made and can be relied upon to stand with certainty for an indefinite period pressures higher than those which I have found it convenient to use. A press permits the employment of pressures which an unarmoured bag could not be expected to withstand, and, constructed in this way, is without prejudice as to the use to which it may be put—whether a periodic or a continuous process—whether filtration, dialysis, or a combination of the two. This is advisable, for such a press as this may well be called upon to do the routine concentration of diphtheria toxin (a continuous pressure dialysis process), that of tetanus toxin (a periodic process—ultrafiltration followed by pressure dialysis), to free tuberculin from glycerol on occasion, and to do any experiments of a similar nature that may be required.

No plates are used in the press—between each pair of membranes and the next on either side are inserted perforated and corrugated material when the press is made up. The celluloid sheet used for keeping apart the lead plates of certain types of accumulators is eminently suitable for the purpose when only low pressures are used. Care must, of course, be taken in filling the press to see that the membranes are not burst by applying pressure before it is full. Although I have done without them in this apparatus, plates with a flat edge but embossed elsewhere with a suitable pyramid pattern would undoubtedly give the advantage of additional security in this respect.

In another apparatus, not illustrated, which I have used as an ultrafilter only, one such plate, ten inches across, covered on each side with a membrane and placed between two flat half-inch steel plates has been used with success. Liquid to be filtered is forced through fine passages in both outer plates and the filtrate runs out from a small hole in the side of that between. The filtering area is 831 sq. cm. and the amount of fluid lost in the apparatus is 2–3 cc., e.g. 50 cc. of blood serum at 2 atmospheres gave in 20 minutes 40 cc. of protein-free filtrate containing sugar and salts but giving no biuret reaction. The residue recovered on gently aspirating at the inlet tubes was 7·5 cc. The filters used were toxin proof, but for the particular experiment quoted much softer and more porous films would have done.

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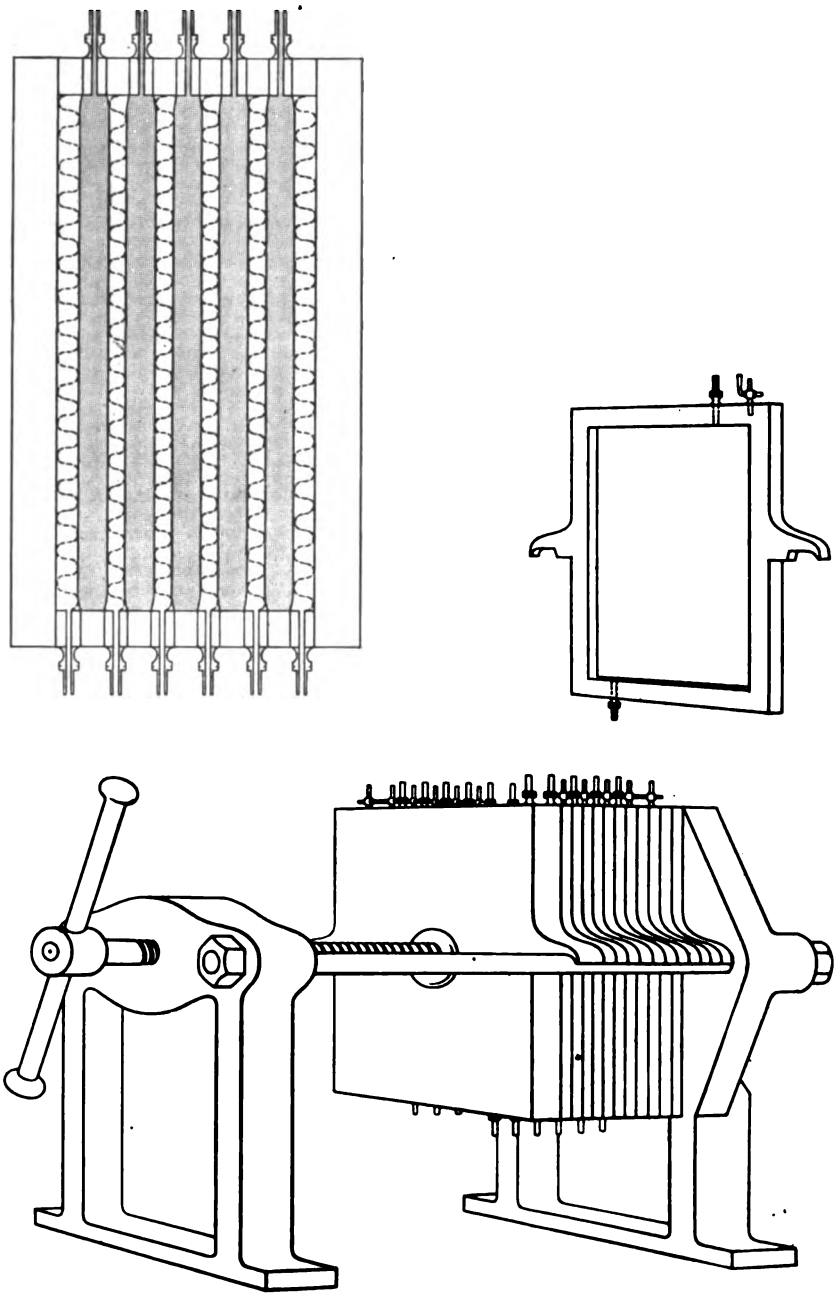

Fig. 4.

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The advantages of working in this fashion with apparatus more suitably designed for the purpose than is possible using glass tubes and rubber stoppers only, are so obvious that this point need not be further emphasised.

General remarks. Although requiring a certain amount of special apparatus to support them so that no liquid to be filtered can escape or get round the film into the filtrate, the flat films offer many advantages over those made in test tubes. They are perfectly uniform, may be characterised quantitatively, and are readily and exactly reproducible at will. Dealing with them involves less trouble, for when a flat film is poured the 200 cc. or so of dilute solution is finished with: but when a bag is made the droppings of thick solution from the inverted tube must be collected again in the bottle, and, on account of evaporation, the composition of the "stock solution" is continually changing. I have examined strips of flat films in a paper testing machine and plotted stress-strain diagrams of them. Their liquid nature, for they continue to stretch at almost constant tension, is, as one would anticipate, particularly marked in those whose main constituent is water. The tensile strength increases remarkably rapidly as "wetness" falls for films of the same nitrocotton content. There is every indication that impermeability to large molecules does the same thing. Unfortunately, experiments designed to test whether tensile strength could be correlated in the same way with m and the average size of the pores could not be continued: though an investigation on these lines could not fail to give instructive results.

In conclusion I would urge that any investigation of porosities of films to proteins should be accompanied by determinations of p_H., and a statement of the electrolyte content of the materials examined, and the nature of the preservative, if any, used. It is now fully recognised how fundamentally these conditions affect the state of aggregation of proteins, and the adsorption of other materials by them.

Summary.

Some notes have been made on test tube shaped bags prepared from collodion, and used for simple ultrafiltration and dialysis experiments. Their lack of uniformity and the difficulty of accurate reproduction militate against their use for careful work. Their circular section is a further disadvantage in that the area of the film operative per unit volume of material treated is large only when the tubes are small, and that, unless the bags be armoured, the pressure that can be used is limited to that which the unsupported membrane can withstand.

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Flat membranes may be made by pouring alcohol-ether solutions of collodion on to levelled plate glass, and then plunging the film, from which alcohol and ether are more or less removed by evaporation, into water. Using this method, and taking certain precautions described, they may be made with remarkable uniformity. By keeping as far as possible to constant conditions, these membranes may be described fully, for purposes of reference and reproduction and as a guide to their properties, by stating the kind of nitrocotton used, the weight of it per sq. cm. they contain, and the "wetness," i.e. the ratio of the weight of a piece of film to its weight when dried to constancy. Films, made in the manner described, containing 8 mg. per sq. cm. of the particular nitrocotton used and weighing, with contained water, 40 mg. per sq. cm., or others where $m = 5$ and $w = 17{\cdot}5$, have been found to retain quantitatively all antigens with which experiments have been made. Practical applications of this result are given in the paper following this [Glenny and Walpole, 1915]. Their permeability to simpler molecules, salts, and water remains high, and filtration through them is rapid.

It is suggested that in these films, more than in other ultrafilters in general use, the channels leading through the gel structure from one side to the other are of a certain uniformity in size. None of them is large enough to allow any antigen to pass; but the structure is highly porous and, because there are many such channels, rapid ultrafiltration results.

Arrangements of apparatus are described whereby films of this type whether flat or in the shape of test tubes may be made and utilised for ultrafiltration and pressure dialysis.

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XXVII. DETECTION AND CONCENTRATION OF ANTIGENS BY ULTRAFILTRATION, PRESSURE DIALYSIS, ETC. WITH SPECIAL REFERENCE TO DIPHTHERIA AND TETANUS TOXINS.

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One has but to know something of the precarious character of routine toxin production to be aware that the artificial conversion of low value toxins to those of high titre is a problem of more than mere academic interest.

In the search for a suitable process by which this may be done it is soon realised that purely chemical methods such as those referred to so completely by Pick [1912] are not available, as they are usually cumbersome and costly, and any effective concentration invariably entails an enormous loss of antigen. Amongst other possibilities the ammonium sulphate method of Heinemann [1908] failed in my hands, possibly because phenol was present; while an alcohol method, applied by Banzhaf, I have not tried on account of expense, for it is essential that any routine process should be simple and cheap. The matter was left at this stage for several years until the accidental discovery was made that on acidification diphtheria toxin, from which salts had been removed by dialysis in a collodion membrane, developed a slight precipitate which contained all the toxin. Moreover, collodion membranes of a special kind [Walpole, 1915] could be made which, though reasonably permeable to water, salts, etc., nevertheless retained quantitatively diphtheria toxin and

[1] A. T. Glenny is responsible for all the tests referring to diphtheria and tetanus toxin purification which require the use of animals; and, also, for the study of the question from the immunological standpoint. The details of physico-chemical technique are supplied by G. S. Walpole.

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all other antigens with which experiments were subsequently tried. These unexpected results, in flat contradiction to those of Baroni [1911] for example, who found diphtheria and tetanus toxins to pass through the collodion membranes he employed, form the basis of the concentration methods here put forward. The various experimental difficulties have disappeared as the result of experience, but, so far, it is only in the case of diphtheria toxin that precipitation by a trace of acid after the removal of the salt can be used as an effective short cut to a purification of the antigen. The invariable result, so far as my experience goes, that antigens do not pass through these membranes suggests their employment not only for routine toxin purification, but in addition as a laboratory test to form a first opinion on the specificity or otherwise of the toxic constituents of any cultural fluid.

The Purification of Diphtheria Toxin.

A batch of diphtheria toxin is appraised by the immunologist by its "binding unit" content per cc., a quantity which for all purposes is most readily measured as the L_+ dose, though this would not necessarily be so if the original media were not similar or had not disintegrated, during the growth of the bacillus, on similar lines. Also, the reservation is made that the "binding units per cc." of these broths are by no means *proportional* to their utility: rather some such scale as the following is taken:

L_+ dose	Use for immunising
0·15 cc.	"excellent"
0·30 cc.	"good"
0·50 cc. and over	"unusable."

In other words poor toxin cannot be made to act as good toxin simply by taking more of it, for experience has shown that the development of antitoxin in the horse is greater when the effective immunising material is interfered with in its action as little as possible by accompanying nitrogenous matter.

It may therefore be taken as a working hypothesis, remembering that the experience of the immunologist is the final and only appeal, that, providing the loss of toxin is minimal, the extent to which the binding units per milligram of nitrogen is increased by this purification process is some sort of measure of its efficiency. When the material accompanying the toxin is so far reduced as to be physiologically of no importance in the dose accompanying the greatest toxin injection given, it is obviously not a true measure because

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further purification serves no useful purpose: it may be inadvisable, in fact, for one would suspect, on the ground of general experience, an increased instability of the toxin as it approaches purity.

The essential process we have employed to concentrate and purify diphtheria toxin is to dialyse it under pressure using a collodion membrane of a particular kind dealt with more fully, for the sake of convenience, in a separate paper [Walpole, 1915]. The dialysed material is then acidified and centrifuged. The precipitate, dissolved in a trace of alkali, constitutes the concentrated material. It should be kept in the cold after the addition of a small quantity, say, 0·3 per cent., of phenol.

To know when the material has dialysed sufficiently to pass on to acidification either of the following methods may be used. A sample may be tested by adding 0·3 cc. N acetic acid to 10 cc. If the precipitate formed flocculates well and leaves a bright supernatant fluid no further dialysis is necessary. This point corresponds, for the broths which have been dealt with, to that where no more colouring matter passes out through the walls of the bag and to a conductivity of about 0·00078, i.e. that of 0·0065 N KCl. For routine work with a continuous apparatus [Walpole, 1915, p. 290] rough conductivity determinations are much the most convenient method for checking the working and determining how much dialysed toxin may be drawn off each day in safety.

Results.

Where this process is applied the cost of toxin production is considerably reduced, for batches are always worth the units they contain, whether they are good or bad, and no toxin however poor need be thrown away. Periodic recurrences of bad toxin to which every laboratory is subjected need, therefore, no longer be feared.

The following generalisations summarise the results of our experience upon which this concentration process for diphtheria toxin had been developed.

1. *Whether by pressure dialysis or ultrafiltration no toxin passes through these membranes.*

These results have been checked so many times that special experiments need not be cited. Part of the evidence supporting them will be found in the ensuing pages. [See also Walpole, 1915, p. 288.]

As a matter of convenience, however, the details of some routine concentrations are given at this juncture, for they depend essentially upon the impermeability of these bags to diphtheria toxin.

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At the time of writing two sets of apparatus [Walpole, 1915, Fig. 3] each consisting of eight bags which were set up some months ago are still in use. One has handled over 100 litres; the other about 44 litres, of which two small batches were worked out more or less completely.

Details of concentration experiments. March 12th to April 1st. Using the continuous apparatus [Walpole, 1915, Fig. 3], 12 litres of diphtheria bouillon, J 2520 (L_+ dose = 0·40 cc.: nitrogen = 2·8 mg. per cc.) gave, in 20 days, 8500 cc. of pressure dialysed material (nitrogen = 1·96 mg. per cc.). The process was not hurried in any way; nothing was drawn off for three days, then 500 cc. per day were collected through the run, and then a couple of days allowed to elapse before all the bags were emptied. The excess of pressure used was 0·3 atmosphere and the consumption of the dialysing fluid, 0·30 per cent. phenol in distilled water, ten litres per day. After acidification the precipitate, representing twelve litres of the original bouillon, was dissolved in alkali and diluted to 250 cc., giving a solution whose L_+ dose was found to be 0·012 cc., and the nitrogen per cc. 2·80 mg.

The result may be roughly stated that, with a loss of 30 per cent., the binding units per mg. nitrogen and also the binding units per cc., have been increased 33 times.

The previous batch of toxin treated by this set of collodion bags was J 2530, of which 11·5 litres (L_+ dose 0·40 cc.: nitrogen 4·6 mg. per cc.) gave 230 cc. of concentrated material (L_+ dose 0·01 cc.: nitrogen 6 mg. per cc.) representing a yield of 80 per cent.

When the same set of bags had handled 50 litres of material whose L_+ dose was 0·4 cc., a sample of dialysate from *C* was collected. They were then lifted clear from the glass jars containing them and some of the ultrafiltrate dripping from them collected (that collected in the first hour or two was, of course, discarded). Neither of these solutions interfered with the increase of weight of the guinea pig when injected in a dose of 1 cc.

The lost toxin, therefore, does not pass through the bags, nor is it to be found in the supernatant fluid above the precipitate produced by acidification. It would seem probable that it represents destruction by light, or by being left at room temperature and in a state of increased purity at a reaction which is probably not that at which it is most stable, at some stage in the process. Experiments on this point are in progress. Should the loss be actually in the filter itself the recovery therefrom of a highly toxic material may be anticipated.

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2. *The toxin goes through the best and thickest parchment commercially obtainable, though the parchment is far less permeable to water than the collodion membrane through which toxin cannot pass.*

A large volume of toxin J 2536 (L_+ dose = 0·35 cc.) filtered through parchment under a slight pressure yielded after some days 50 cc. of filtrate resembling the original in colour and general precipitation reactions. The L_+ dose of the filtrate was 0·60 cc. This is a striking example of the fact that low permeability of a membrane to water is not necessarily accompanied by low permeability to toxin. Using the same batch of toxin and a collodion membrane ($m = 5$: $w = 3{\cdot}5$, [see Walpole, 1915]) no toxin could be detected in the filtrate (1 cc. nil), though the volume of the filtrate per square centimetre per hour was many times greater in this case. Below are given details of a repetition of this experiment, using another sample of the parchment and another collodion bag.

Collodion bag.	Parchment bag.
Area exposed 260 sq. cm. Time 24 hours. Filtrate 8 cc., of which 1 cc. has no effect on the guinea pig. Pressure 6″ water.	Area exposed 1960 sq. cm. Time 24 hours. Filtrate 6 cc. of which 0·1 cc. kills a guinea pig. Pressure 7″ water.

This point is, in our opinion, worth full and patient investigation because of its far-reaching consequences in bacteriological work generally.

3. *The amount of toxin in a cultural filtrate bears no relation to the amount of precipitate obtained on acidification after pressure dialysis through these membranes.*

4. *The method is universally applicable at least to all brews of diphtheria toxin of the type made in these laboratories.*

Samples of 200 cc. each from twenty-nine different brews of toxin were dialysed, each in a separate bag, under pressure for four days against distilled water changed daily. Colouring matter had then ceased to come out through the bags and the specific conductivity of the inside fluid was in every case less than 78×10^{-5}. All gave nicely flocculated precipitates on adding 6 cc. of 1·0 N acetic acid to the contents of each bag. They were placed in order of the amounts of their precipitates as well as could be judged by eye. The first fifteen precipitates were then mixed and dissolved in 60 cc. of distilled water. Equal volumes of the corresponding original brews were also mixed. Both solutions were tested for "binding units" with the following results.

The first fifteen samples amounted in volume to 3000 cc. (L_+ dose = 0·45), from which were obtained 60 cc. of concentrated material (L_+ dose = 0·012).

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The results referring to the remaining fourteen samples which all gave smaller precipitates when treated in the same way may be summarised:

2800 cc. (L_+ dose = 0·45) gave 56 cc. (L_+ dose = 0·012).

Though no other attempts were made solely to settle these points they are confirmed by the general experience of numerous other concentrations. The yield in both the above cases is 80 per cent., and the concentrations of the binding units per unit volume fifty times.

5. *If the cultural filtrate after pressure dialysis is only partially precipitated by a trace of acid, and if, after removing this small precipitate, it is completely freed from precipitable matter by further acidification, then nearly all the toxin will be found in the first precipitate.*

Four litres of a mixture of toxins (L_+ dose = 0·40: nitrogen 4·63 mg. per cc.) gave as first fraction material which, dissolved in a trace of alkali, was diluted to 80 cc. The L_+ dose was 0·008 cc. and the nitrogen content 3·5 mg. per cc. in this solution. The second fraction, comprising the rest of the precipitable material, when similarly treated gave 80 cc. of a solution of L_+ dose 0·30 cc. and nitrogen content 6·02 mg. per cc. Hence the yield of binding units on the first precipitate is as near as one can tell quantitative, and that on the second, which contains nearly twice as much nitrogen, is only 2 per cent. of the total binding units originally taken.

This experiment was performed either to confirm or throw doubt upon a previous result in which the distinction between the binding unit content per mg. nitrogen of successive fractions was equally marked.

6. *There is no evidence that this routine concentration process brings about any separation of the toxic from the binding elements of the cultural filtrate.*

The following two experiments were made with a batch of toxin J 2538, the L_+ dose and M.L.D. of which were 0·38 cc. and 0·005 cc. respectively. In both of them the concentrations of L_+ dose and M.L.D. were, within the experimental error, equal.

Experiment 1. 200 cc. of original toxin yielded a trace of precipitate which was dissolved in a convenient volume, which amounted to 16·6 cc. The L_+ dose of this solution was 0·05 cc. and M.L.D. 0·0007 cc. It is seen that both the toxicity and the binding units have been concentrated to about the same extent, i.e. 7·1 times in the one case and 7·6 times in the other.

Experiment 2. 1800 cc. of original toxin yielded 28 cc. of concentrated toxin having L_+ dose 0·008 cc. and M.L.D. 0·0001 cc. The concentrations are, in this case, forty-eight times for the L_+ dose and fifty times for the toxicity.

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7. *Some change occurs in the material undergoing pressure dialysis which is not reversible, for it is impossible to reconstruct the original material by adding to the fluid remaining in the bag a corresponding amount of dialysate after concentration to the correct volume.*

The original material gives no precipitate on acidification: the reconstructed material does, i.e. it appears as if the salt when once removed fails to inhibit precipitation by acid when again added.

500 cc. of diphtheria toxin, brew Y 76, gave 5 litres of pressure dialysate (A) against 0·3 per cent. phenol, with a reduction in volume to 180 cc. (B). Comparative tests showed that the addition of 2 per cent. sodium chloride reduced by only very little the precipitate obtained by acidification. Similarly, if 170 cc. of A concentrated ten times by evaporation be added to 9 cc. of B, almost a full precipitation still occurs.

The immunising value of concentrated diphtheria toxin.

In the light of experience it is easy to establish whether any particular toxin has good immunising value or not, but to demonstrate statistically the comparative values of two toxins would require many horses owing to their individual variations.

Two normal horses immunised with concentrated toxin prepared from material so poor in value as to be unusable reached 800 A.T. units and 1400 A.T. units per cc. Individual horses immunised with unconcentrated toxin have reached 2000 A.T. units per cc., but it requires more than usually good toxin to reach an antitoxic unit content of even 1400 per cc. The average value of horses under similar treatment with good unconcentrated toxin was 700 units per cc.

The details of the response to treatment, although referring to two horses only, indicate clearly that concentrated toxin is at least as good as unconcentrated toxin unit for unit for purposes of immunisation. The volume of the injections was very small, though the number of binding units injected was the same as that usually given when unconcentrated toxin was used. The general condition of both the horses was far better than that of horses immunised in the ordinary way.

Another horse that had received several immunisations of unconcentrated toxin was gradually falling off in unit value. After a course of concentrated toxin, in which the largest dose given was 10 cc., higher value sera were obtained from it than had been obtained at any time during the previous treatment.

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These experiments on the immunising value of concentrated diphtheria toxin, as compared with that not so concentrated, are being continued.

Purification of Tetanus Toxin.

Owing to the vigorous digestion of the broth constituents by the tetanus organism the concentration of toxin per mg. nitrogen obtained by ultrafiltration followed by pressure dialysis is in this case greater even than in that of diphtheria cultural filtrates. The rapidity of filtration is, too, for the same reason, much greater. The slight precipitate obtained on acidifying the dialysed material is, as has been already stated, not toxic. This curious difference between diphtheria and tetanus toxins must be of considerable theoretical significance.

In the tests given below the animal used was the mouse.

Experiment 1. An experiment has been described [Walpole, 1915, p. 288] where comparative tests of the porosities of two bags showed that no toxin could be detected in one of the filtrates and very little in the other. A dose of 0·0005 cc. of the original material placed in the bag caused death on the 4th day.

Experiment 2. Toxin BDC (0·0005 cc. death on 4th day); 30 cc. were concentrated to one-seventh the volume with continual change of water outside, and diluted again to its original volume: no change in M.L.D. could be detected. A small precipitate obtained by acidifying and centrifuging an aliquot portion was found to be non-toxic.

Experiment 3. 190 cc. of toxin BDC were reduced to 6 cc. as above. Of this

0·00005 cc. gave death on the 3rd day
0·00002 cc. „ „ 7th „

that is to say, that the toxin was concentrated about twenty times and the volume reduced to one-thirtieth.

Experiment 4. Toxin BDA (L_+ dose 0·05 cc.); 10 cc. of this were reduced to about 3 cc. and then made up to the original volume with physiological saline solution. The L_+ dose was found to be unaltered.

Experiment 5. 10 litres toxin BKA (1 cc. contains 3·7 mg. nitrogen) gave in the apparatus described by Walpole [1915, Fig. 2] in six days 9630 cc. of ultrafiltrate divided between five bags. Tests on these filtrates showed that one bag only allowed a detectable amount of toxin to pass through (M.L.D. 0·4 cc.). Twenty-four hours' pressure dialysis followed, and then

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300 cc. of concentrated and purified toxin containing 3 mg. of nitrogen per cc. were collected from inside the bags.

It would seem from these observations that the horse, while still receiving the full measure of specific antigen, may be spared 97 per cent. of the nitrogen and, in this case all the indole, fatty acids, etc., of a particularly noxious and evil-smelling fluid.

As in the case of diphtheria toxin the cost and labour of the production of the tetanus antigen may be reduced by this means, for the value for immunising purposes of a given toxin is that of the number of units it contains. Also, a greater certainty in immunising horses and again a saving in the cost of horses and their keep during immunisation may be confidently anticipated.

Experiments with other Cultural Filtrates.

It has already been suggested that one of these membranes might be used to form a first opinion as to the specificity or otherwise of the toxic element of any cultural fluid.

A toxic filtrate from a culture of the malignant oedema bacillus (Besson) grown in these laboratories by Mr Buxton was found to give an ultrafiltrate of undiminished toxicity. This observation indicated the probability that the toxic substance was not of the nature of a specific antigen. The filtrate in question was further investigated by Barger and Dale, who found that its essential toxicity was due to the presence of a high concentration of ammonia[1].

On the other hand, the toxic constituent of a broth culture of an organism producing a fatal epidemic in the squirrel was found to be retained by one of these bags. The suspicion that this would be a specific antigen was confirmed by Mr Buxton, who is investigating the outbreak, for he was able, using the toxin in its concentrated and purified state, to produce a corresponding antitoxin in the goat. Pressure dialysis applied to tuberculin gave in the two cases tried a complete removal of the glycerol without detectable loss of antigen. Mr Buxton, to whom I am again indebted for animal tests, employed Koch's method, using sensitised guinea pigs. For ophthalmic tests where the presence of glycerol is not permissible, or for low potency tuberculins where further concentration by evaporation is impossible, the method should prove to be of considerable utility in view of the waste and inconvenience of the alcohol precipitation method at present practised.

Mallein has also been concentrated and freed from glycerol in the same way.

[1] Private communication by Drs Barger and Dale.

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Filtration of pituitary active principle, etc.

Although they are not antigens, the behaviour of secretin and the active principle of pituitary extract when filtered under pressure through these bags is of considerable interest. A simple pituitary extract gave a filtrate which, compared with the original solution and the residue, showed that although much of the active principle had passed through it had not done so at the same rate as the water, salts, etc. A similar result was obtained with the co-enzyme of zymase, in an experiment in conjunction with Prof. Harden, to whom I am indebted for the material and the tests.

Dr P. P. Laidlaw has communicated to me privately that one of his secretin preparations, filtered through a standard bag into saturated picric acid, gave at once a crystalline precipitate of marked activity.

I wish to express my indebtedness to the assistance of Mr Ralph Defries during the course of this work.

Summary.

Experiments are described in which the permeability of collodion-water membranes of a special type to various antigens was investigated. Owing to the fact that none of the antigens tried was found to pass through these membranes their use is suggested for the purpose of forming a first opinion as to the specificity or otherwise of the toxic element of any cultural fluid. The recognition and subsequent concentration of the antigen of a fatal epidemic in the squirrel was effected by this means, and an investigation of the nature of the toxicity of filtrates of malignant oedema cultures led to its complete elucidation.

Mallein and tuberculin were freed from glycerol and a considerable quantity of nitrogenous material in this way.

A process for the concentration and purification of diphtheria toxin which at a yield of 70–80 per cent. diminishes the nitrogen content about 50 times has been fully worked out. It is performed by dialysis under pressure, followed by acidification, centrifugalisation, and re-solution of the small precipitate obtained in a trace of alkali.

If a continual supply of toxin having L_+ dose 0·20 could be relied upon, this artificial conversion of low value toxin to that of high titre, would remain more of academic than practical interest. But, the majority of batches do not reach this high level and hence this concentration

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process is of considerable importance. One thing is certain and that is that the periodic recurrence of "bad" toxin to which every laboratory is subjected need now no longer be feared.

By ultrafiltration followed by pressure dialysis a concentration of tetanus toxin may be effected which should be of considerable practical value, for the disintegration products of the broth materials which accompany the antigen are particularly noxious in this case.

These membranes are impermeable to enzymes, but allow to pass secretin, the pituitary active principle, the co-enzyme of zymase, and the toxic constituent of Witte peptone.

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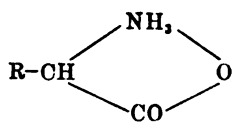
XXVIII. NOTE ON TYROSINE.

By ARTHUR GEAKE and MAXMILIAN NIERENSTEIN.

From the Biochemical Laboratory, Chemical Department, University of Bristol.

(*Received June 9th, 1915.*)

Reference has already been made to a preliminary observation that tyrosine is not methylated by diazomethane in ethereal suspension [Geake and Nierenstein, 1914, 1, p. 292]. The present communication confirms this observation, which is of interest both for the chemistry of methylo-caseinogen [Geake and Nierenstein, 1914, 1], since this substance gives Millon's reaction, and for that of tyrosine. The fact that the carboxyl group of tyrosine is not methylated is quite in accordance with our experiences with other amino acids [Geake and Nierenstein, 1914, 2] which have led us to accept the "betaine" formula [Meyer and Jacobson, 1913]



for amino acids. It has further also been found that an hydroxyl-group in para-position to a nitrogen atom is not methylated by diazomethane, as for example in the cases of γ-pyridone, chelidamic and cuminaminic acids [Pechmann, 1895, Meyer, 1906, 1 and 2]. These substances yield unchanged γ-pyridone, the dimethyl ester of chelidamic acid and the methyl ester of methoxy-cuminaminic acid.

Meyer expresses the influence of the nitrogen atom on the hydroxyl group by dotted lines, but emphasises at the same time as follows: "das Wesen der Erscheinungen wird aber natürlich durch derlei Zeichensprache nicht im mindestens enthüllt und man kann nur den in jüngster Zeit wiederholt

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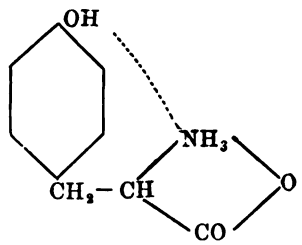
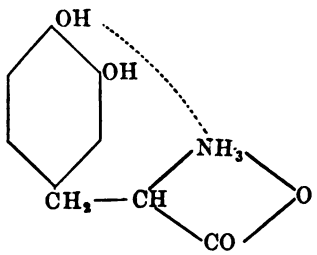
gezogenen Schluss rekapitulieren, dass die derzeit herrschenden Anschauungen über Valenz nicht ausreichen, die Gesamtheit der Beobachtungen zu erklären." In this connection reference ought also to be made to the classical investigations of Kaufmann [1911] on valency with which our observations are in full accord.

The following formula therefore expresses the behaviour of tyrosine towards diazomethane:



In view of the fact that the hydroxyl group in the meta-position of cuminaminic acid undergoes methylation when treated with diazomethane an experiment was also made with 3:4-dihydroxyphenylalanine [Funk, 1911] to which the formula:



is assigned. The reaction was accompanied by a strong evolution of nitrogen, which indicated that apparently methylation had taken place, especially as nitrogen is not evolved when tyrosine is treated with diazomethane. The material kindly sent by Dr Funk was, however, so small in quantity that we were not able to isolate any definite product. We intend to repeat the experiment on a larger scale later on, since dihydroxyphenylalanine is now more accessible through its isolation from *Vicia faba* by Guggenheim [1913].

Since the carboxyl groups of the amino acids and the peptides become accessible to methylation on acylation, when the "betaine"-ring is opened [Geake and Nierenstein, 1914, 2], the action of diazomethane on glycyl-tyrosine was also investigated especially with the view of studying the behaviour of the tyrosine hydroxyls in caseinogen towards diazomethane. The product thus obtained was the glycyl-tyrosine methyl ester, as we were

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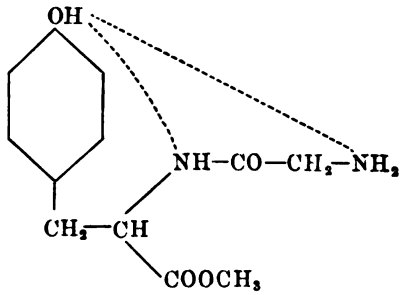
able to recover on hydrolysis glycyl-tyrosine and on oxydation *p*-hydroxybenzoic acid. It is apparent that the nitrogen atoms of the glycyl-tyrosine



continue to have an influence on the hydroxyl-group, which leads us, at least for the time-being, to the conclusion *that the tyrosine hydroxyls in methylo-caseinogen are not methylated by diazomethane.*

Experimental.

Action of diazomethane on l-*tyrosine.* One g. tyrosine from hair (m.p. 303–306°) was treated with 0·5 g. (2 mol.) diazomethane; no visible reaction took place and 0·85 g. unchanged tyrosine (m.p. 303°) was recovered. In another experiment 0·2 g. tyrosine from caseinogen and 0·1 g. (2 mol.) diazomethane were used and 0·2 g. unchanged tyrosine (m.p. 306°) recovered.

Action of diazomethane on dl-3 : 4-*dihydroxyphenylalanine.* 0·2 g. dihydroxyphenylalanine was treated with 0·25 g. (4 mol.) diazomethane. The substance dissolved with evolution of gas, leaving a residue (0·04 g.) which after recrystallisation from alcohol and water formed small needles which melted at 225°. It is possible that this was unchanged dihydroxyphenylalanine for which the following melting points are given: 238° (Funk) and 280° (Guggenheim). The ethereal solution gave an oil which we have not investigated.

Action of diazomethane on glycyl-l-*tyrosine.* One g. glycyl-*l*-tyrosine prepared according to Abderhalden and Oppler [1907] was treated with 1 g. diazomethane; on evaporation of the ether a solid was left, which crystallised from methyl alcohol in small needles and melted at 123–124°. The *glycyl*-l-*tyrosine methyl ester* thus obtained dissolves easily in alcohol, acetone and to some extent also in water. If the ester is melted, cooled and then remelted the melting point rises to about 295–300°, glycyl-*l*-tyrosine anhydride being formed during the melting process as in the case of leucyl-*l*-tyrosine [Fischer and Schrauth, 1907].

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Analysis: 0·1488 g.: 13·2 cc. N_2 (moist) 19°, 772 mm.
0·3038 g.: 27·3 cc. N_2 (moist) 16°, 770 mm.
0·1206 g.: 0·2546 g. CO_2, 0·0722 g. H_2O.
0·1222 g.: 0·2558 g. CO_2, 0·0672 g. H_2O.

		Cal. for $C_{12}H_{16}O_4N_2$
C ..	57·60, 57·09 %	C .. 57·14 %
H ..	(8·43), 7·06 %	H .. 6·35 %
N ..	10·32, 10·58 %	N .. 10·94 %

*Hydrolysis of glycyl-*l*-tyrosine methyl ester.* One g. of the ester was dissolved in 10 cc. alcohol and warmed on the water bath with 2·5 cc. N/10 alcoholic potash. It was then diluted with 10 cc. water and acidified with 2·5 cc. N/10 hydrochloric acid and the solid filtered off. The product consisted of glycyl-*l*-tyrosine and glycyl-*l*-tyrosine anhydride, which were separated by shaking with dilute hydrochloric acid. The anhydride was crystallised from water and melted at 293–297°. The glycyl-*l*-tyrosine recovered from the hydrochloride crystallised from dilute alcohol in needles, melting at 183°, the melting point not being depressed by glycyl-*l*-tyrosine; mixed melting point found, 182–183°.

*Oxidation of glycyl-*l*-tyrosine methyl ester.* Two g. of the ester were dissolved in 20 cc. of 30 per cent. potassium hydroxide and boiled for 3–4 hours with an excess of potassium permanganate. When acidified with hydrochloric acid a small precipitate was obtained which after several recrystallisations from water formed small prismatic needles, melting at 213–215° (m.p. of *p*-hydroxybenzoic acid, 213–215°). A mixed melting with this acid gave 213–215°. For further identification 0·3 g. of the oxidation product was also converted with dimethyl sulphate and alcoholic potash into anisic acid (m.p. found, 186°; m.p. of anisic acid, 184°).

In conclusion we wish to thank Dr Casimir Funk for the *dl*-3 : 4-dihydroxyphenylalanine kindly sent us and also the Bristol University Colston Research Committee for a grant which defrayed the expenses of this investigation.

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XXIX. THE ORIGIN OF THE GLUCOSAMINE OBTAINED IN THE HYDROLYSIS OF *BOLETUS EDULIS.*

By WINIFRED ROSS (*Carnegie Scholar*).

From the Chemical Research Laboratories, United College of St Salvator and St Leonard, University of St Andrews.

(*Received June 22nd, 1915.*)

The existence of chitin in fungus cellulose has long been known, but the question of the origin of the glucosamine obtained in the hydrolysis of dried fungus extract has given rise to two distinct views. The first view is that the glucosamine residue is pre-existent, either as glucoproteins, or as glucosamine glucosides. It has been proved by Müller [1901] that glucoproteins give on hydrolysis a carbohydrate compound identical with that obtained from chitin. The second theory is, that glucosamine is an adventitious product, formed in the process of hydrolysis by the interaction of carbohydrate residues and amino acids.

From the results of numerous experiments, strong evidence in favour of the first theory has been obtained. In particular, reference must be made to the recent isolation of definite glucosamine glucosides by Yotake and Sera [1912]. The material used in their investigations was a species of Lycoperdon, (*L. gemmatum, Batsch*), and, by hydrolysis with concentrated mineral acid, they obtained a compound, termed by them "lycoperdin," which from its reactions proved to be an amino glucoside containing two glucosamine residues. Still more recently, they obtained the same compound from other species of Lycoperdon (*L. bovista*), and also from *Geaster granulosus*.

With the object of isolating similar glucosides, the following research was carried out on *Boletus edulis*. This particular fungus was selected, as it was already known that a large proportion of fungus cellulose existed in the plant, and, therefore, it seemed a probable source of amino glucosides.

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Investigations on *Boletus edulis* had previously been carried out by Reuter [1912], who examined various extracts, but more particularly those yielding amino acids. He finally obtained a fraction containing the carbohydrate residues, but did not examine it in detail. It was accordingly decided to examine this carbohydrate fraction for the presence of amino glucosides.

In the extraction of the fungus material, Reuter's experimental method was carefully followed, and thereafter the examination of the dried extract was carried out according to the process of Yotake and Sera [1912]. Hydrolysis was effected by sulphuric acid, and phosphotungstic acid used to precipitate amino compounds. Owing to a slight solubility of certain of these compounds in phosphotungstic acid, both precipitate and filtrate were examined. Three amorphous products were obtained (as described in the experimental part). These were optically active and contained both carbohydrate and amino groups. From the filtrate a compound was isolated which showed reducing properties, but on hydrolysis the power of reduction practically disappeared. The remaining two products showed no sign of reduction either before or after hydrolysis. The examination thus gave no trace of glucosamine glucosides or of complexes containing glucosamine. The substances actually isolated appear to be condensation compounds of sugars with amino acids.

The absence of glucosamine from these compounds indicates that in *Boletus edulis* the amino sugar does not occur in the form of an amino glucoside, as otherwise glucosamine would necessarily have been obtained by these methods.

There remained, however, the possibility that the extraction of the carbohydrate fraction of *Boletus edulis* by means of sulphuric acid had been incomplete, and accordingly the undissolved residue was further examined. It was treated with concentrated hydrochloric acid, and kept just below boiling point for seven or eight hours. The insoluble material was filtered, and the solution concentrated and examined for glucosamine. Again no trace of this compound was found.

As however Reuter [1912] had obtained a definite amount of glucosamine from the carbohydrate fraction of *Boletus*, another hydrolysis was carried out on the original material, using on this occasion concentrated hydrochloric acid. The result confirmed Reuter's experience, as glucosamine hydrochloride was isolated in amount practically identical with that obtained by him.

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These observations seem on first inspection contradictory, but they indicate that the production of glucosamine from *Boletus edulis* must be due to the formation of the amino sugar during the process of hydrolysis with concentrated hydrochloric acid. Hydrolysis with sulphuric acid yields a solution and a residue, neither of which contains glucosamine compounds. Since it is known that the action of concentrated sulphuric acid on chitin gives various results, in some cases entirely removing the amino group as ammonia, the absence of glucosamine in the products isolated might be accounted for in that way.

The work of Yotake and Sera [1912] however makes this seem improbable, as they isolated amino glucosides from reactions in which concentrated sulphuric acid was used. The conclusion arrived at therefore, is, that the glucosamine isolated from *Boletus edulis* under certain conditions is derived from a complex, of the nature of a glucoprotein, containing carbohydrate and amino acid residues, but does not exist in the form of a glucosamine glucoside.

This idea may be formulated as follows:

$$\begin{array}{c|c} | & | \\ CH\cdot NH\cdot CO\cdot CH\ldots(I), & \xrightarrow{H_2O} \quad CH\cdot OH + NH_2\cdot CO\cdot CH\ldots(II), \\ | & | \end{array}$$

$$\text{or} \quad \begin{array}{c|c} | & | \\ CH\cdot NH_2 + OH\cdot CO\cdot CH\ldots(III), \\ | & | \end{array}$$

where (I) represents one characteristic grouping in a glucoprotein, which hydrolyses in two alternative ways, according to the hydrolyst used, giving rise to cleavage products (II) (a sugar and an acid amide); or to the products (III) (an amino sugar and an acid). Subsequent hydrolytic action on products (II) would yield ammonia and a carboxylic acid.

Experimental.

The *Boletus edulis* was supplied specially for the purpose of this research by Messrs Caesar and Loretz, of Halle, and the extraction of the material was carried out by Burroughs, Wellcome & Co., according to Reuter's method [1912]. The total weight received by them was 3565 grams, but after drying and grinding this was reduced to 3266 grams. The different extracts were:

(*a*)	Ether giving	72·3 grams
(*b*)	Alcohol ,, ...	1061·2 grams
(*c*)	Water ,, ...	785·3 grams

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From aqueous washings 71·5 grams were obtained. The remaining undissolved matter weighed 1040 grams.

The material used in the research was the dried powdered residue left after extraction with water. As the method employed involved the manipulation of solutions of very large bulk, experiments were carried out on the scale described below, and since the products isolated were not fully examined and therefore cannot be definitely named, a system of lettering has been adopted to avoid confusion.

70 grams of the dried residue were ground in a mortar with 70 cc. of concentrated sulphuric acid. A thick paste was formed to which 350 cc. of water were added. The mixture was transferred to a large flask and boiled under a reflux condenser for six hours. The deep brown solution was filtered from the undissolved residue containing humic acid salts; the latter was again extracted with dilute sulphuric acid, boiled for some time, and the liquid filtered, leaving the undissolved *Residue A*.

The joint filtrates were then partially neutralised by the addition of a cold saturated solution of barium hydroxide, in order to remove 95 per cent. of the sulphuric acid present. The precipitate was filtered and the brown filtrate decolourised by boiling with charcoal. After being allowed to stand for forty-eight hours the solution was again filtered and 10 per cent. phosphotungstic acid solution added until precipitation was complete. From this, *Precipitate B* and *Filtrate B* were obtained. Before precipitation with phosphotungstic acid, polarimetric examination of the clear solution showed that it possessed a slight but perceptible dextrorotation, and, after precipitation, *Filtrate B* still showed optical activity.

Examination of Precipitate B. The light brown precipitate of phosphotungstates was washed by continued decantation with 5 per cent. sulphuric acid, filtered, rubbed with dilute sulphuric acid and again filtered. Finally, it was dissolved in dilute acid, leaving a dark residue (*Residue D*) which was filtered, and the clear solution was then treated with barium hydroxide until only a slight acidity remained. Barium sulphate was removed by filtering, the solution was saturated with carbon dioxide, and again filtered. In order to remove the last traces of barium, dilute sulphuric acid was cautiously added until a very slight acid reaction was produced. The clear solution was concentrated on the water bath at 30°–40° under diminished pressure, and when reduced to very small bulk, was further concentrated in vacuum until a thick syrup remained. On the addition of absolute alcohol, a white

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precipitate was obtained (*Precipitate C*), which was filtered, washed with absolute alcohol, and dried.

Examination of Precipitate C. The product obtained was an amorphous yellow powder, deliquescent, insoluble in alcohol and ether. The melting point was somewhat indefinite (46°–50°), and the compound showed no action on Fehling's solution either before or after hydrolysis. Nitrogen was present in the form of amino nitrogen as indicated by the reaction with nitrous acid. Traces of carbohydrate were also present as shown by Molisch's test. In aqueous solution the compound showed a very slight rotation, $[\alpha]_D = +4{\cdot}1°$. As it was evident that this product contained no glucosamine residue, it was not further examined.

Examination of Residue D. The dark-brown undissolved material from recrystallisation of the phosphotungstic acid precipitate was dissolved in water, and treated in the same way as *Precipitate C.* It was similarly decomposed by barium hydroxide and, after filtering, the highly coloured filtrate was saturated with carbon dioxide, made slightly acid with dilute sulphuric acid, and concentrated in vacuum to a thick syrup. Precipitation with absolute alcohol gave a dark brown, amorphous, deliquescent powder. The product contained amino nitrogen, but showed no sign of reduction with Fehling's solution either before or after hydrolysis with concentrated hydrochloric acid, so that glucosamine complexes were absent. It gave no biuret reaction.

Examination of Filtrate B. The phosphotungstic acid filtrate was neutralised with barium hydroxide, the precipitate filtered and the solution concentrated on the water bath at 40° and finally in vacuum until a thick syrup was obtained. From this, on precipitation with absolute alcohol, a white product separated, which was filtered, washed with absolute alcohol and dried (*Precipitate E*).

Examination of Precipitate E. The substance was a white amorphous powder, soluble in water, insoluble in alcohol and ether. Ignition on platinum gave an inorganic residue consisting of barium salts. The compound contained amino nitrogen, readily reduced Fehling's solution, and in aqueous solution showed a slight rotation. Unsuccessful attempts were made to remove the inorganic matter. Part of it was precipitated as barium sulphate, but even after further purification barium salts still remained. The percentage of barium was therefore estimated and the product analysed.

Melting point 78°–80° (indefinite). The substance darkened slightly, and began to char at 110°.

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Analysis of Purified Precipitate E.

Barium	12·43 %
Carbon	37·84 %
Hydrogen	6·97 %
Amino Nitrogen	2·325 %
Total Nitrogen	2·719 %
Rotation (in water) $[\alpha]_D$	− 13·3°

Hydrolysis with concentrated hydrochloric acid gave a yellow crystalline product which showed no reducing properties.

The high percentage of barium indicates that the compound is a salt, moreover the action on Fehling's solution observed before hydrolysis indicates that a carbohydrate group is present, and the fact that the total nitrogen is combined as amino nitrogen is important. It would thus appear that the compound is the barium salt of a carbohydrate acid combined with amino residues. These amino groups cannot however be attached to the penultimate position in the sugar chain, as otherwise glucosamine would have been produced on complete hydrolysis. This has already been shown from the results obtained by Irvine, Thomson and Garrett [1913], and by Fischer [1902].

Examination of undissolved Residue A. Since none of the products isolated showed any trace of the presence of glucosamine, *Residue A* was examined. It was shaken with concentrated hydrochloric acid for twelve hours, and then boiled gently under a reflux condenser for six hours. The undissolved material was filtered, and the dark-brown solution concentrated on the water bath under diminished pressure. No crystals of glucosamine hydrochloride separated, and precipitation with alcohol gave a slight dark-brown precipitate which showed no sign of reduction with Fehling's solution.

The barium sulphate precipitate obtained before precipitation with phosphotungstic acid was similarly extracted, and gave the same result.

As this process is the standard method for preparing glucosamine, it is evident that derivatives of the amino sugar could not be present.

Hydrolysis of the Original Extract with Concentrated Hydrochloric Acid.

50 grams of the original extract were treated with 600 cc. of concentrated hydrochloric acid, and boiled under a condenser for seven hours. The solution was diluted and filtered, and then concentrated on the water bath at 50°. A crystalline product separated, which was filtered, dried, and recrystallised

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from water. It readily reduced Fehling's solution, and contained amino nitrogen.

Permanent rotation of the compound in water, $[\alpha]_D = +71{\cdot}5°$.
The amino nitrogen was estimated and found to be 7·567 %.
Glucosamine hydrochloride requires $[\alpha]_D = +72{\cdot}5°$; Amino nitrogen = 7·86 %.

The substance was undoubtedly glucosamine hydrochloride. The total yield of the recrystallised product was 1·7 grams which corresponds with the amount obtained by Reuter [1912] from a similar process of hydrolysis.

The author desires to express her indebtedness to Professor J. C. Irvine of St Andrews University for suggesting the topic of this research and for the interest he has taken in it; also to Dr W. N. Haworth for his supervision of the work.

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XXX. PRELIMINARY NOTE ON THE PREPARATION OF COLLODION DIALYSERS OF GRADED PERMEABILITY.

By WILLIAM BROWN.

From the Department of Plant Physiology and Pathology, Imperial College of Science and Technology, London.

(*Received July 6th, 1915.*)

In a recent publication, Walpole [1915] has described a method by which the permeability of collodion membranes is specified in terms of (1) the weight of dry nitro-cotton per sq. cm., (2) the "wetness" of the membrane.

In a series of experiments which have been in progress for some time, the author has found that collodion dialysers, constant in character and of a wide range of permeability, can be prepared by treating air-dried collodion membranes with varying percentages of alcohol in water. After such treatment for a suitable time, the membranes are thoroughly washed in water, and are then ready for use. The permeability of such membranes is directly related to the strength of the alcohol employed, so that by the use of various concentrations, membranes of almost any degree of permeability may be obtained: from those which will keep back e.g. copper sulphate, to those which are permeable to such a highly colloidal substance as aniline blue.

A second method of preparing graded dialysers which depends upon a process of differential drying has also been worked out. A full account of these researches will shortly be forthcoming.

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XXXI. A SIMPLE APPARATUS FOR FILTRATION UNDER DIMINISHED PRESSURE.

By JAMES COLQUHOUN IRVINE.

From the Chemical Research Laboratory, United College of St Salvator and St Leonard, University of St Andrews.

(*Received July 7th, 1915.*)

The separation of small quantities of crystalline matter from syrupy solutions, and, conversely, the recovery of residual syrups from semi-crystalline products, are common experimental difficulties. For work of this nature, the filtering tube shown below may be substituted with advantage for the ordinary filtering flask which, particularly when small quantities of material have to be manipulated, is inconvenient and involves undue experimental loss.

As the apparatus has not only served its original purpose well but has also found general application in filtrations under diminished pressure and in analytical work involving the Gooch crucible, a short note on its use may not be out of place, although it presents no very novel features.

The apparatus consists of a cylindrical tube furnished with a delivery stop-cock *C* at the lower end, the upper part being expanded into the bulb *A*. The side-limb carries a three-way stop-cock *D* and permits of connection to the pump. The filter funnel (or Gooch crucible) is attached as shown to the filter tube, which is supported by a clamp about *B*. When in use, *C* is closed and *D* opened to the pump and, on completing the filtration or when the tube is filled, the filtrate can be run off by admitting air to the apparatus by means of *D* and thereafter opening *C*.

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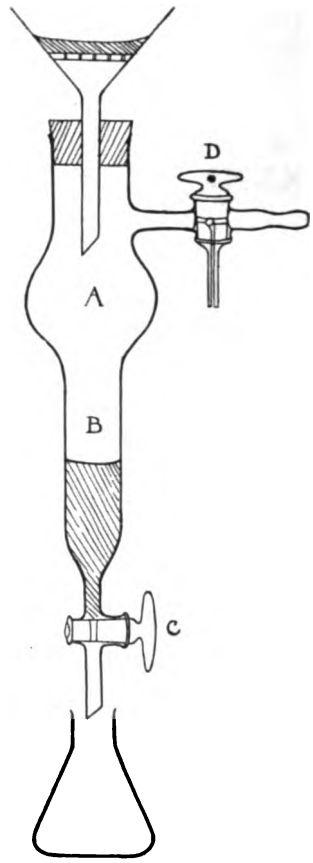
Many advantages may be claimed for the arrangement. Syrupy liquids can be drained from the delivery tap without much loss and, as the apparatus can be enclosed in a hot-air bath, the filtration of viscous or saturated solutions is greatly facilitated. It is unnecessary while emptying the tube to remove the funnel or to detach the connection with the pump, which is kept running continuously. The objectionable practice (inevitable when a conical filtering flask is used) of pouring pure liquids out of the neck, into which the cork carrying the funnel is afterwards inserted, is avoided. When used in analytical work in conjunction with a Gooch crucible, the removal of washings for testing purposes is very conveniently done.



Practice with the apparatus reveals other and less obvious advantages, such as the facility with which the economical washing of a filtered residue by alternate slow filtration at ordinary pressure and rapid draining in a vacuum may be carried out, and also the ease with which the last traces of syrupy filtrates may be washed from the tube. This is best done by partially closing *D* to the pump and opening *C*. Air then enters by the delivery tap and bubbles rapidly through the solvent, thereby effecting thorough mixing and solution of the adhering syrup. A double purpose is served by the expansion *A*, as, in addition to adding to the capacity of the tube, it prevents liquids which boil under diminished pressure from rushing over into the side-limb, the bubbles being broken on rising to the bulb.

The diagram is drawn to scale ($\frac{1}{4}$ natural size) and gives the measurements which have been found most suitable for analytical work, or for filtrations of average scale. With the dimensions figured, 150 cc. of filtrate can be collected without emptying the tube, while the maximum and minimum sizes used allow of filtration of 300 cc. and 20 cc. respectively in each operation.

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XXXII. THE RELATION OF SALIVARY TO GASTRIC DIGESTION.

By LESLIE ALGERNON IVAN MAXWELL

From the Physiological Laboratory, University of Melbourne.

(*Received July 22nd, 1915.*)

Introduction.

The exact significance of the presence of ptyalin in the saliva has been a matter of dispute.

That an enzyme should be secreted in the mouth to be presently destroyed in the stomach, whilst a more efficient diastase is present in the secretion of the pancreas, is rather curious. The researches of Edkins on gastric secretin, however, show that dextrins may act as stimulants to the formation of this hormone. Since dextrin is one of the products of hydrolysis of starch resulting from the action of ptyalin, we can assign at least one valuable function to this enzyme. It occurred to me that another important function might on theoretical grounds be assigned to this ferment. Hedin [1906, 1912] has shown that charcoal and certain powders have the power of adsorbing enzymes, thereby hindering their activity. It is logical to suppose that colloidal starch solutions might adsorb pepsin and hence inhibit the activity of this enzyme. Saliva by hydrolysing the starch might prevent the adsorption of pepsin by the colloidal carbohydrate and would thus aid gastric digestion. The following experiments were conducted in order to elucidate this question.

Method.

Pepsin was allowed to act on fibrin in the presence of either colloidal starch solutions or starchy foods and the rate of digestion compared with that which occurred after previous hydrolysis of the carbohydrate by saliva

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or takadiastase. The Grützner method was used to determine the rate of digestion, a solution of carmine serving as a standard colour against which the digestion tubes were compared. The tubes were placed in a shaker (to avoid errors due to diffusion) within a thermostat at 39° C.

In the various experiments pepsin was placed in contact with carbohydrate material for some time in order that adsorption might take place; this interval of time is indicated throughout the paper by the word *contact*.

The following solutions were prepared:

(1) A 2 per cent. starch solution.

(2) A 2 per cent. solution of takadiastase.

(3) 0·25 g. pepsin (Merck) dissolved in 100 cc. of 0·3 per cent. HCl.

The diastase solution was divided into two equal portions, one of which was boiled to destroy the ferment. Saliva was used in many experiments.

Exp. 1. *A.* 4 cc. unhydrolysed starch + 1 cc. pepsin HCl.

B. 4 cc. hydrolysed starch + 1 cc. pepsin HCl.

Contact 150 min. Equal quantities of fibrin were now added.

Fibrin digestion. *A* required 13 min.; *B* required 55 min.

There is evidently a marked difference in the activity of the pepsin after being in contact with colloidal starch.

In this experiment the contact was 150 min., which will be shown in the next experiment to be unnecessarily long.

Exp. 2. *Boiled Starch.*

A. 1 cc. pepsin HCl + 4 cc. hydrolysed starch.

B. 1 cc. pepsin HCl + 4 cc. unhydrolysed starch.

Unboiled Starch.

C. 1 cc. pepsin HCl + 4 cc. (starch + diastase).

D. 1 cc. pepsin HCl + 4 cc. (starch + boiled diastase).

Contact 20 min. Equal quantities of fibrin were then added.

Fibrin digestion. *A* required 13 min.; *B* required 60 min.; *C* required 13 min.; *D* required 13 min.

It is evident that:

(*a*) Adsorption of pepsin is very marked in 20 min.

(*b*) Unboiled, i.e. non-colloidal starch suspensions do not hinder peptic digestion.

Exp. 3. Soluble starch was prepared by treating pure potato starch with 7·5 per cent. HCl in thermostat at 40° until the resultant gave a clear solution

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in warm water and a blue reaction with iodine. The acid was removed by washing with water to which alcohol had been added.

Erythrodextrin was not specially prepared for this experiment but a dextrin supplied by Merck, giving a rich red colour with iodine, was used. Saliva was substituted for takadiastase in all further experiments—except where otherwise indicated.

The carbohydrate solutions were all 2 per cent.

A. Unboiled starch suspension.
B. Unhydrolysed starch solution.
C. Hydrolysed starch solution.
D. Soluble starch.
E. Erythrodextrin.

3 cc. of each solution were taken and 1 cc. boiled filtered saliva added to each of tubes *A*, *B*, *D*, and *E*. 1 cc. unboiled saliva added to *C*. 1 cc. pepsin HCl added to *A*, *B*, *C*, *D*, *E*. Contact 30 min. Equal quantities of fibrin were then added.

Fibrin digestion. Time in min.

	A	*B*	*C*	*D*	*E*
I.	10	49	10	32	10
II.	10	50	10	35	14

It is evident from these results that soluble starch causes a certain amount of adsorption, but that the power is not retained by the erythrodextrin molecule. Unboiled starch, as in the previous experiment, did not exhibit any adsorptive effects.

Exp. 4. Further evidence as to the adsorption of pepsin by starch solutions is given by the following figures:

A. 1 cc. 0·25 per cent. pepsin solution + 3 cc. hydrolysed starch (2 per cent.).

B. 1 cc. 0·25 per cent. pepsin solution + 3 cc. unhydrolysed starch (2 per cent.).

Added equal quantities of fibrin to each. Contact 21 min. Removed fibrin and quickly washed with water. Added 5 cc. 0·3 per cent. HCl to *A* and to *B*.

Fibrin digestion. *A* required 15 min.; *B* required 46 min.

Another experiment showed that colloidal starch solution had little adsorptive effect upon HCl.

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Exp. 5. A further test was performed to see if the presence of maltose might act as a catalyst to the action of pepsin HCl. Tubes were arranged as follows:

A. 4 cc. water (distilled).

B. 4 cc. 2 per cent. maltose.

C. 3 cc. water + 1 cc. saliva.

To each was added 1 cc. pepsin HCl. Contact 30 min. Equal quantities of fibrin were then added.

Fibrin digestion. *A* required 9 min.; *B* required 9 min.; *C* required 9 min.

Maltose has no catalytic effect upon pepsin HCl digestion and therefore cannot account for the difference between the rates of digestion of fibrin in the presence of unhydrolysed and hydrolysed starch.

Experimental work up to this juncture had been confined to pure starch solutions or the derivatives of starch. The experiments were next extended to certain food stuffs.

Exp. 6. *Maizena.* A preparation of maizena was made by adding the material to boiling water.

Tube *A.* 4 cc. maizena + 1 cc. boiled saliva.

Tube *B.* 4 cc. maizena + 1 cc. unboiled saliva.

Contact with saliva 60 min. *A* blue with iodine. *B* no colour with iodine.

1 cc. pepsin HCl added to each. Contact 20 min. Equal quantities fibrin then added.

Fibrin digestion. *A* required 30 min.; *B* required 12 min.

By the time that *A* had acquired the colour of the standard solution the fibrin in *B* had almost completely disappeared, although there was of course a considerable amount of fibrin still undigested in *A*.

Exp. 7. *Rice.* Boiled rice ground in a mortar. Equal weighed quantities placed in tubes *A* and *B*.

2 cc. boiled saliva added to *A*.

2 cc. unboiled saliva added to *B*.

Contact with saliva 60 min.

Added to each 1 cc. pepsin HCl. Contact 35 min. Fibrin was then added.

Fibrin digestion. *A* required 37 min.; *B* required 17 min.

Microscopical examination of material from tubes *A* and *B* after one hour's contact with saliva showed the following appearances:

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A. Particles of comparatively massive appearance (as compared with those in *B*), staining darkly with iodine.

B. Particles of feathery disintegrated nature, giving a very slight stain or none whatever with iodine.

Exp. 8*a*. *Potato*. Potato boiled for three-quarters of an hour. Equal weighed quantities taken.

A. Potato + 2 cc. boiled saliva.

B. Potato + 2 cc. unboiled saliva.

Contact with saliva 45 min.

Added 1 cc. pepsin HCl to each. Contact 23 min.

Added 2 cc. 0·3 per cent. HCl to make total volume = 5 cc. and then added fibrin.

Fibrin digestion. *A* required 45 min.; *B* required 35 min.

Microscopical examination of iodine stained specimens of *A* and *B* showed that colloidal starch was still present within the cells of *A*, whereas it had been dissolved completely out of the cells of *B*.

Exp. 8*b*. The experiment was repeated using potato which had been boiled for two hours and had a very mashed appearance. Iodine stained specimens showed much cell debris. The cell contents had disappeared in many cases, leaving the skeleton of cellulose unstained.

Equal quantities *A* and *B* were taken as before and treated in exactly the same manner as in the previous experiment except that the contact with pepsin HCl was 20 min.

Fibrin digestion. *A* required 42 min.; *B* required 30 min.

It will be noted that potato exercises a comparatively small inhibitory effect upon peptic digestion.

This is probably accounted for by the fact that much of the colloidal starch still remains enclosed within the potato cells and hence does not exert an adsorptive effect upon pepsin.

Exp. 9. *Porridge*. Porridge ground in mortar to uniform consistency. Equal weighed quantities taken.

A. Porridge + 2 cc. boiled saliva.

B. Porridge + 2 cc. unboiled saliva.

Contact with saliva 120 min.

Added 1 cc. pepsin HCl. Contact 15 min.

Added 2 cc. 0·3 per cent. HCl to make total volume 5 cc., and then added fibrin.

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Fibrin digestion. *A* required 29 min.; *B* required 10 min.

Treatment with iodine and microscopical examination showed that the starch had completely disappeared from *B*, whereas it was abundant in *A*. Disintegrated cells were present in both preparations.

Exp. 10. *Bread.* Bread was finely ground in a mortar. Equal weighed quantities taken and treated exactly as in Experiment 9, with the exception that salivary digestion occupied 70 min. and the contact with pepsin HCl was 30 min.

Fibrin digestion. *A* required 45 min.; *B* required 28 min.

Exp. 11. *Gum Acacia.* A 4 per cent. solution of gum acacia in water was made.

A. 3 cc. gum acacia + 1 cc. boiled saliva.

B. 3 cc. gum acacia + 1 cc. unboiled saliva.

Contact with saliva 75 min.

Added 1 cc. pepsin HCl. Contact 30 min. Fibrin was then added.

Fibrin digestion. After 65 min. there was equality in the liberation of colour in *A* and *B*, but both tubes were far below the standard tube in depth of colour.

The experiment was repeated with the following modifications:

A. 3 cc. gum acacia + 1 cc. saliva + 1 cc. pepsin HCl. Contact 35 min. Added fibrin.

B. 1 cc. pepsin at 39° for 35 min. Then added fibrin, followed immediately by 3 cc. gum acacia + 1 cc. saliva.

C. 1 cc. pepsin kept at 39° for 35 min. Then added 3 cc. gum acacia + 1 cc. saliva followed immediately by fibrin.

Fibrin digestion. After 90 min. there was equal liberation of colour in the three tubes, but still far from the standard colour tube. It appears that saliva does not alter the adsorptive power of gum acacia, and further that this adsorption is very complete and takes place almost instantaneously.

Exp. 12. *Biscuit.* A plain starchy biscuit was powdered in a mortar and equal quantities weighed out.

Microscopical examination of an iodine stained specimen revealed the fact that most of the starch grains were unburst. Equal quantities of taka-diastase (2 per cent. solution) boiled and unboiled respectively were added to tubes *A* and *B*. Contact with diastase 30 min.

No appreciable change—discernible to the eye—had taken place at the

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end of this interval, and the enzyme was therefore allowed to act all night —4.25 p.m. to 9.30 a.m.

1 cc. pepsin HCl added to *A* and *B*. Contact 20 min. Fibrin was then added.

Fibrin digestion. *A* required 47 min.; *B* required 32 min.

Owing to the large amount of material undissolved which modified the colour of the liberated carmine, it was difficult to satisfy oneself as to the exact end point.

Starch grains were abundant in *A*, even after prolonged contact with diastase.

It is interesting to note in connection with the foregoing experiments that in the cat, dog, horse, sheep and ox the saliva is non-amylolytic. The last three animals consume large quantities of starchy food stuffs. The starch grains however are intact—unless the food has been artificially prepared by cooking, steaming, etc.—and do not form colloidal solutions and hence do not hinder gastric digestion.

Conclusions.

1. Peptic digestion is delayed in the presence of colloidal starch solutions through adsorption of enzyme, the time interval being increased four-fold in the presence of 2 per cent. starch solution.

2. There is a stage in the disruption of the starch molecule at which the phenomenon of adsorption of pepsin is lost. This occurs between the amylo-dextrin and erythro-dextrin stage.

3. Unboiled starch does not hinder the action of pepsin, a factor of importance in the dietetics of herbivora.

4. Cooked farinaceous foods—rice, potato, bread, porridge, maizena—all hinder peptic digestion if not first subjected to salivary digestion.

5. Gum acacia causes marked inhibition of peptic activity, and this is not prevented by previous contact with saliva.

6. The saliva of man, in virtue of its ptyalin, plays a considerable part in aiding gastric digestion by hydrolysing colloidal starch, which would otherwise adsorb pepsin.

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XXXIII. THE REDUCING ENZYMES OF DRIED YEAST (LEBEDEFF) AND OF RABBIT MUSCLE.

By ARTHUR HARDEN and ROLAND VICTOR NORRIS.

From the Biochemical Department, Lister Institute.

(*Received August 11th, 1915.*)

In a previous communication [Harden and Norris, 1914] it was shown that the power of reducing methylene blue is restored to dried yeast (Lebedeff), which has been washed until it has been rendered inactive in this respect, by a number of substances (e.g. salicylaldehyde) and the suggestion was made that these substances were capable of acting as acceptors for the oxygen activated during the reduction process. On the other hand many easily oxidisable substances (e.g. quinol) are without effect.

Further qualitative experiments carried out in a similar manner to those previously described have shown that comparatively few substances are able to act as acceptors in this way. The following list gives the results so far obtained, including those previously mentioned.

Positive.

Salicylaldehyde	Dihydroxyacetone	Bouillon
Benzaldehyde	Glycollic acid	Boiled yeast extract
Anisaldehyde	Citric acid	Normal horse serum
Isovaleraldehyde	Glyceric acid	Sterile milk
Phloroglucinol	Succinic acid	

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Negative.

Quinol	Glucose	Creatine
p-Phenylene diamine	Laevulose	Asparagine
Pyrogallol	Glycocoll	Guanidine-acetic acid
Resorcinol	Alanine	Guanidine nitrate
Citral	Tyrosine	Glycol
Glycerol	Malic acid	Witte peptone
Propylene glycol	Tartaric acid	Acetone
2-3-Butylene glycol	Mandelic acid	Pyruvic acid
Formic acid	Yeast nucleic acid	Methylglyoxal
Acetaldehyde		

Of the hydroxy acids examined, lactic acid, either as free acid or sodium salt, was very active, whilst glycollic, glyceric and citric acid produced a very slow reduction. Sodium succinate also gave a very slow reduction.

Owing to their special interest the cases of acetaldehyde (negative) and lactic acid (positive) were more closely investigated.

Acetaldehyde. As acetaldehyde might be expected to exert some inhibitory action on the enzyme concerned in the reduction, special experiments were first made to ascertain how far this was the case.

Exp. 1. Four flasks were set up, each containing 5 cc. of a 10 per cent. suspension of the washed yeast (Lebedeff). To these were added the following solutions, and the mixtures incubated for two hours at 37°.

	Water cc.	Methylene Blue cc.	Boiled Yeast Washings cc.	1 per cent. Acetaldehyde cc.	Result
1.	6	1	0	0	—
2	1	1	5	0	Reduced in 49 min.
3.	5	1	0	1	—
4.	0	1	5	1	Reduced in 78 min.

In this experiment therefore the acetaldehyde has a pronounced inhibitory effect. When the concentration is halved however this inhibition ceases to be perceptible.

Exp. 2. A similar experiment was made with half the concentration of acetaldehyde.

	Water cc.	Methylene Blue cc.	Boiled Yeast Washings cc.	1 per cent. Acetaldehyde cc.	Result
1.	6·5	0·5	0	0	—
2.	1·5	0·5	5	0	Reduced in 21 min.
3.	6	0·5	0	0·5	—
4.	1	0·5	5	0·5	Reduced in 21 min

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That the amount of aldehyde here employed is sufficient to reduce the methylene blue is proved by the fact that in presence of washed muscle (p. 335) half this amount produced complete reduction.

A further experiment with varying amounts of acetaldehyde also gave completely negative results.

Exp. 3. 10 cc. of a 10 per cent. suspension of washed yeast (Lebedeff) were used, together with the following additions.

	Saline cc.	Methylene Blue cc.	1 per cent. Acetaldehyde cc.	Result
1.	5	0·5	0	—
2.	3	0·5	2	—
3.	4	0·5	1	—
4.	4·5	0·5	0·5	—
5.	4·75	0·5	0·25	—
6.	4·9	0·5	0·1	—

Lactic Acid. Both free lactic acid and sodium lactate were found to have a positive action, and attempts were made to establish some quantitative relation between the amounts of methylene blue reduced and the amount of lactic acid oxidised and also to ascertain the nature of the products formed from the lactic acid.

1. No completely satisfactory solution of the former of these two problems was attained.

Any oxygen present in the various solutions interferes with this estimation by reoxidising the leuco-methylene blue and thus increasing the amount of lactic acid required for the reduction of a given weight of the colouring matter. Complete removal of the oxygen was very difficult to effect, especially from the yeast suspension, but the partial removal attained by boiling out the water and other solutions which were used and working in an atmosphere of nitrogen caused a great acceleration of reduction and a fall in the ratio of lactic acid to methylene blue.

Exp. 4. A solution of methylene blue was prepared containing 0·4 mg. per cc., i.e. 0·001 molar.

10 cc. of a 10 per cent. suspension of the washed yeast were taken in each case, along with 1 cc. N/100 lactic acid and 1 cc. of the methylene blue solution. The yeast suspension was made with water saturated with nitrogen.

(*a*) The solutions of methylene blue and lactic acid were boiled up and cooled in nitrogen, and the yeast suspension then added. Reduction occurred in 7 min.

(*b*) The solutions were boiled up and cooled in air and the yeast suspension then added. Reduction occurred in 21 min.

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Exp. 5. Working with air free solutions in an atmosphere of nitrogen, the following results were obtained.

10 cc. of a 10 per cent. yeast suspension and 1 cc. 0·01 N lactic acid were used, the total volume being made to 16 cc. with water. Temperature 37°.

Methylene Blue cc.	Time for Reduction min.
2	19
3	43·5
4	68
5	90

The greatest amount of methylene blue reduced by 1 cc. of 0·01 N lactic acid was 5 cc., the molecular equivalent being 10 cc.

The interesting observation was made that the reduction in certain cases was greatly accelerated by exposure to light. This only occurred with solutions containing dissolved oxygen, and was ultimately traced to the accelerating influence of light on the reoxidation of leuco-methylene blue by oxygen, a reaction which is being further investigated.

2. *Products of oxidation of lactic acid.* The distillate from a mixture of the washed dried yeast, methylene blue and lactate gave a pronounced reaction for acetaldehyde. This suggested that the lactic acid was first converted by oxidation into pyruvic acid. The latter would then be decomposed by the carboxylase of the washed yeast into carbon dioxide and acetaldehyde, which, being incapable of further oxidation under the conditions of the experiment, could be detected in the liquid. Quantitative experiments showed that a considerable amount of acetaldehyde was produced. This amount however was considerably less than the equivalent of the lactic acid destroyed, and it is to be supposed either that a considerable proportion of the acetaldehyde condenses to alcohol or disappears in some other way, or that only a part of the lactic acid oxidised is converted into pyruvic acid. A special experiment showed that none of the aldehyde was converted into alcohol, there being somewhat more found in the absence than in the presence of methylene blue.

Pyruvic acid itself could not be detected.

Exp. 6. 20 g. of dried yeast were washed and made to 50 cc. and two mixtures made:

A. 25 cc. yeast suspension + 25 cc. sodium lactate solution (approx. N/5) + 0·4 g. methylene blue.

B. 25 cc. yeast suspension + 25 cc. sodium lactate solution.

These were incubated at 37° for 3·75 hours, at the end of which the methylene blue in *A* was completely reduced. The two mixtures were separately

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distilled in steam to remove aldehyde, 55 cc. of distillate, cooled by ice and salt, being taken. To the residue oxalic acid was added, and the liquid then extracted with ether in a continuous apparatus, the ether distilled off, the residual mixture of oxalic and lactic acids boiled with calcium carbonate. The calcium oxalate and excess of carbonate were filtered off, washed and the calcium estimated in the filtrate and taken as equivalent to lactic acid.

The first distillate from *A* gave a strong Rimini reaction for acetaldehyde, whilst that from *B* was negative to this test.

The amount of acetaldehyde was in this case estimated by adding *p*-nitrophenylhydrazine dissolved in acetic acid and weighing the hydrazone, a control being carried out with water and the weight of precipitate subtracted.

Results.	Residual lactic acid in *A*	0·282 g.
	,, ,, *B*	0·482 g.
	Loss of lactic acid	0·200 g.

Acetaldehyde nitrophenylhydrazone, *A–B*, 0·1111 g., corresponding to 0·0273 g. acetaldehyde.

This amount of acetaldehyde is molecularly equivalent to 0·0558 g. lactic acid. Hence in the reduction of 0·4 g. of methylene blue, 0·2 g. of lactic acid has disappeared and at least 0·0558 of this has been converted into acetaldehyde.

Exp. 7. A second experiment carried out with larger quantities gave similar results. In this case the aldehyde was estimated by the bisulphite method (treatment with bisulphite and titration of the excess by iodine). An aliquot portion of each distillate was tested for alcohol by boiling with silver oxide to remove aldehyde, distilling and estimating the alcohol by the method of Nicloux.

40 g. dried yeast were washed and made to 270 cc. 100 cc. of this suspension, 100 cc. of water and 50 cc. of a sodium lactate solution were taken in each case, 1·6 g. of methylene blue being added to *A*, but not to *B*.

Reduction occurred after 5·5 hours at 37°.

Results.	Residual lactic acid in *B*	1·235 g.
	,, ,, ,, *A*	0·415 g.
	Loss of lactic acid	0·820 g.
	Aldehyde produced in *A*	0·058 g.
	Lactic acid equivalent of aldehyde	0·118 g.
	Alcohol in *A*	0·025 g.
	,, ,, *B*	0·098 g.

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Hence in the reduction of 1·6 g. of methylene blue, 0·8 g. lactic acid has disappeared and at least 0·118 of this has been converted into acetaldehyde.

Preliminary experiments with rabbit muscle.

When the minced muscle of the rabbit is washed several times with saline, it behaves in a similar manner to dried yeast, and loses the power, possessed by the normal muscle, of reducing methylene blue.

The experiments with muscle are not however as satisfactory as those with yeast. When the muscle is very finely divided, the enyzme is removed as well as the acceptor. On the other hand, when the division is not so fine certain portions retain the acceptor and local reduction occurs. In any case the removal of the acceptor is not so complete as with dried yeast and reduction occurs in the controls in about 2–3 hours.

The chief interest in the results so far obtained with muscle lies in the fact that specific differences exist between the activating action of certain substances on the enzyme in muscle and on that in yeast. Thus acetaldehyde reactivates washed muscle, whereas, as shown above, it has no effect on washed yeast. Hitherto only qualitative experiments have been made with muscle and the positive results obtained are given below. The experiments are being continued.

Exp. 8. The muscle was minced and then washed with normal saline three times. Portions of 5 g. were then weighed out, a given volume of the substance to be tested and of the methylene blue solution, and if necessary of saline, added and the whole well shaken and incubated at 37°.

Substance tested	Vol. cc.	Methylene Blue cc.	Saline cc.	Time required for reduction at 37°, minutes		
Boiled muscle washings	5	0·5	—	14		
Boiled yeast washings	5	0·5	—	12·5		
Sodium lactate, 5 per cent.	1	1·0	8		28	
Acetaldehyde, 1 per cent.	1	0·5	9			41
,, ,,	0·25	0·5	9·75			46
Control	—	—	—	– in 50	– in 120	– in 60

None of the substances used reduced the methylene blue in the absence of the muscle.

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Summary.

1. The power of reducing methylene blue is restored to washed dried yeast (Lebedeff) by a number of oxidisable substances but not by others.

2. Lactic acid restores the power of reduction and is itself oxidised in the reaction, yielding acetaldehyde; the amount found however is not equivalent to the lactic acid lost. The lactic acid is probably first converted into pyruvic acid, which is then decomposed by the carboxylase of the yeast into acetaldehyde and carbon dioxide.

3. Rabbit muscle, like dried yeast, when washed loses the power of reducing methylene blue.

4. This power is restored to washed muscle by various substances. The enzyme concerned seems to be specifically different from that present in yeast, since acetaldehyde restores the power of reduction to washed muscle but not to washed dried yeast.

REFERENCE.

Harden and Norris (1914), *Biochem. J.* 8, 100.

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XXXIV. THE SUPPOSED SYNTHESIS OF URIC ACID FROM ITS DECOMPOSITION PRODUCTS BY TISSUE EXTRACTS.

By HENRY MICHAEL SPIERS, *Benn W. Levy Student.*

From the Biochemical Laboratory, University of Cambridge.

(*Received August 10th, 1915.*)

It has long been known that extracts of the organs of the lower animals possess the power of destroying uric acid [Stockvis, 1860; Brunton, 1905; Wiener, 1899, 1902, 1903]. Of the various decomposition products which have been reported at one time or another, allantoin has been shown by Wiechowski [1907] to be the real one, and this observer has demonstrated the quantitative conversion of the acid into this substance by the agency of an active ferment preparation from dog's liver. Ackroyd [1911] obtained the same result on perfusing surviving rabbit's liver with Ringer's solution containing dissolved sodium urate. Bezzola, Izar, and Preti [1909] found that uric acid dissolved in defibrinated ox blood was destroyed by perfusion through surviving dog's liver, and maintained that the uric acid was regenerated on saturating with carbon dioxide the solution containing the decomposition products and again passing it through the organ. Ascoli and Izar [1908] also demonstrated such a re-synthesis of uric acid from its decomposition products in the case of extracts of ox liver. When ox liver extract to which was added sodium urate was aërated and incubated, the uric acid was destroyed, but on saturating the resulting mixture with an inert gas such as carbon dioxide, hydrogen, or nitrogen, or by simply incubating the product in a closed vessel, the uric acid was quantitatively regenerated. Later, the same observers [1909] set out to determine what

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substances could give rise to uric acid under these conditions. Having unsuccessfully tried several substances, allantoin among them, they found that dialuric acid and urea combined to form uric acid in presence of ox liver extract under anaërobic conditions.

The present paper is a repetition of their work. Their technique was employed in the preparation of the extract and during the incubation, but the uric acid was estimated by Hopkins' method, and not by the Ludwig-Salkowski silver method. The liver was obtained fresh from the slaughter-house, and was finely minced and digested for one hour with Ringer's solution or with 0·85 % sodium chloride solution in an incubator, followed by shaking for one hour. It was then strained through muslin. A solution containing a known weight of uric acid was added, together with 1 per cent. chloroform and 1 per cent. toluene, and the flask containing the mixture was placed in a thermostat at 37° and a current of air drawn through. At the end of the incubation, two equal volumes of the liquid were taken, and the uric acid determined in one of them at once. The second portion was then placed in a flask with more chloroform and toluene, stoppered, and again incubated, after which the uric acid was estimated. The proteins were coagulated in presence of dilute acetic acid in the heat, and the coagulum was filtered off, transferred back to the basin, boiled out with water and again filtered, the process being again repeated. To the filtrate and washings, ammonium chloride was added to the extent of 20–22 per cent. and 2 cc. per cent. of strong ammonia. The ammonium urate was decomposed with hydrochloric acid and titrated as in Hopkins' process.

The author was unable to show any difference in the uric acid content of the fluid before and after the anaërobic conditions, but found that the amount of uric acid in the latter case was equal to that which had not been oxidised by the uricolytic ferment in the extract. Therefore the results of Ascoli and Izar were not confirmed.

Experiment I. 500 g. of finely minced ox liver in 2000 cc. of Ringer's solution was maintained for one hour at 37°, and was then shaken for one hour. It was strained through muslin and 1 per cent. chloroform and 1 per cent. toluene added. A solution of 1·5608 g. of uric acid in 87 cc. of 0·1914 N lithium carbonate and water to 250 cc. were added and air was drawn through for 67 hours at 37°. Total volume was 2225 cc. after this period. Two portions *A* and *B* were then taken. *A* equalled 556 cc., *B* was 1124 cc. *A* was coagulated at once; *B* after anaërobic incubation at 37° for 114 hours with further addition of 1 per cent. chloroform and 1 per cent. toluene

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was divided into two equal portions and the uric acid determined in one of them.

	Found	Calculated
A	Uric acid = 0·1957 g.	
B	Uric acid = 0·1824 g.	$\frac{1}{4} \times 1{\cdot}5608 = 0{\cdot}3902$ g.

Experiment II. 250 g. of dog's liver in 1000 cc. Ringer's solution was treated as before. 1·1882 g. of uric acid dissolved in 74 cc. of 0·1914 N lithium carbonate solution made up to 250 cc. were added. Air current for 45 hours at 40°. Final volume was 1230 cc. Two portions *A* and *B* were taken, each of 500 cc. *A* was coagulated at once, *B* after 72 hours of anaërobic incubation at 37° with a further portion of chloroform and toluene.

	Found	Calculated
A	Uric acid = 0·1282 g.	
B	Uric acid = 0·1050 g.	$\frac{500}{1230} \times 1{\cdot}1882 = 0{\cdot}4833$ g.

Experiment III. 347 g. of ox liver in 1500 cc. of Ringer's solution was treated as above. A solution of 1·0118 g. of uric acid in 60 cc. of 0·1914 N lithium carbonate solution and water to 250 cc. were added. Air current 42 hours at 37°. Final volume was 1800 cc. Two portions *A* and *B* were taken, each of 450 cc. *A* was coagulated at once, *B* after 72 hours of anaërobic incubation at 37° with a further addition of chloroform and toluene.

	Found	Calculated
A	Uric acid = 0·0791 g.	
B	Uric acid = 0·0435 g.	$\frac{1}{4} \times 1{\cdot}0118$ g. $= 0{\cdot}2529$ g.

Experiment IV. 350 g. of ox liver in 1750 cc. of 0·85 per cent. sodium chloride solution was treated in the same way. A solution of 1·8155 g. of Merck's monobasic sodium urate (= 1·4120 g. of uric acid) in 240 cc. water and 10 cc. N NaOH solution were added, and air drawn through for 41 hours at 37°; a further 15 cc. of N NaOH was added after 19 hours as the reaction was acid. Two portions *A* and *B* were taken, each equal to a fourth of the mixture. *A* was coagulated at once, *B* after 46 hours of anaërobic incubation at 37° with a further addition of chloroform and toluene.

	Found	Calculated
A	Uric acid = 0·1326 g.	
B	Uric acid = 0·1426 g.	$\frac{1}{4} \times 1{\cdot}4120 = 0{\cdot}3530$ g.

Experiment V. 330 g. of ox liver in 1500 cc. of 0·85 per cent. sodium chloride solution was treated as usual. 25 cc. of N NaOH and a solution of 1·6013 g. of sodium urate (= 1·2454 g. of uric acid) in 500 cc. of water were added. Air stream for 42 hours at 37°. Two portions *A* and *B* were

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taken, each equal to one-fourth of the total. *A* was coagulated at once, *B* after 27·5 hours of anaërobic incubation at 37° with further addition of chloroform and toluene.

	Found	Calculated
A	Uric acid = 0·0498 g.	
B	Uric acid = 0·0530 g.	$\frac{1}{4} \times 1{\cdot}2454 = 0{\cdot}3114$ g.

In the attempted synthesis of uric acid from dialuric acid and urea, two Erlenmeyer flasks with ground-glass stoppers were partially filled with equal volumes of liver extract. In one of them was placed a solution containing 2·0 g. of dialuric acid and 0·5 g. of urea dissolved in 0·85 per cent. sodium chloride solution. In the other, an equal volume of 0·85 per cent. sodium chloride solution was placed. To the contents of each flask 1 per cent. chloroform and 1 per cent. toluene were added. The flasks were thus filled to within 25 cc. of their total volume. They were stoppered and incubated for some days at 37°. The flasks were vigorously shaken twice a day. After the incubation, the contents were coagulated, and an endeavour was made to estimate the uric acid. This, however, was not found, as the following experiments show.

Experiment VI. 250 g. of ox liver and 1000 cc. of 0·85 per cent. sodium chloride solution as before. In flask *A* were placed 500 cc. of the extract together with 75 cc. of 0·85 per cent. sodium chloride solution and 1 per cent. of chloroform and toluene. In flask *B* were placed 500 cc. of the extract and a solution of 2·0 g. of dialuric acid and 0·5 g. of urea in 75 cc. of 0·85 per cent. sodium chloride solution, and 1 per cent. of chloroform and toluene. Incubation period was 220 hours at 37°. The contents were then coagulated.

A No precipitate with ammonium chloride and ammonia.

B No precipitate with ammonium chloride and ammonia.

Experiment VII. Similar to last, but incubation period was 92 hours.

A Very slight precipitate of ammonium urate.

B No precipitate of ammonium urate.

Experiment VIII. Similar to the above, but the mixture was made with 180 g. of ox liver and 1000 cc. of 0·85 per cent. sodium chloride solution, and the incubation was for 70 hours. Again no precipitate was obtained in either case.

I am unable to explain my failure to repeat the work of the Italian investigators. Their technique, so far as their published statements allow, was adopted in all essential details except in the method of estimating uric acid.

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That the ammonium chloride method is reliable for the estimation of uric acid in tissue extracts is shown by the following observations upon human liver, which, as Walter Jones and his co-workers have shown, contains no uricolytic ferment.

Experiment IX. To each of two mixtures, *A* and *B*, containing 250 g. human liver in 250 cc. Ringer's solution, a solution of 0·6050 g. of uric acid in sodium carbonate and 250 cc. Ringer's solution were added.

A Coagulated at once gave 0·6055 g. of uric acid by the ammonium chloride process.

B Coagulated after six hours' aëration gave 0·6075 g.

Experiment X. A similar experiment, but in each case a solution of 0·7050 g. of uric acid in sodium carbonate and Ringer's solution to 250 cc. were added.

A Coagulated at once gave 0·6995 g. of uric acid.

B Coagulated after six hours' aëration gave 0·6980 g.

Summary.

The experiments of Ascoli and Izar were repeated, but no uric acid could be shown to have been formed:

(1) From the decomposition products of uric acid under the influence of ox liver extracts under anaërobic conditions;

(2) From dialuric acid and urea under the influence of ox liver extracts with exclusion of air.

I wish to express my sincere thanks to Professor Hopkins for suggesting this research and for his kind interest and advice.

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XXXV. THE PREPARATION AND COMPOSITION OF CASEINOGEN.

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Definition. In this paper the word *caseinogen* denotes the main protein present in fresh milk; *acidic caseinogen* the protein precipitated from milk by acid; and *casein* the protein precipitated from milk by the action of proteolytic ferments and calcium salts. The experimental facts upon which this terminology is based are set forward in the succeeding pages.

Introduction. The experiments described in this paper were made on caseinogen prepared directly from fresh milk by alcohol precipitation. A considerable quantity of work has been done on the protein precipitated from milk by the addition of acid to it (acidic caseinogen) on the assumption that this substance is the basic protein of milk. The prevalent hypothesis as to the relation of caseinogen to acidic caseinogen is founded upon an experiment of Ringer [1890] in which he showed that when a suspension of acidic caseinogen in water is triturated with calcium carbonate, the protein is dissolved forming a milk white solution. This experimental result is explained on the assumption that acidic caseinogen is an acid which reacts with calcium carbonate forming calcium caseinogenate (assumed to be identical with the caseinogen of milk) and liberating carbon dioxide thus:

$$\text{Acidic caseinogen} + CaCO_3 = \underset{\text{(Calcium caseinogenate)}}{\text{Caseinogen}} + CO_2.$$

The precipitation of acidic caseinogen from milk by the addition of small quantities of acid is expressed by the equation

$$\underset{\text{(Ca caseinogenate)}}{\text{Caseinogen}} + 2H\,\bar{A} = Ca\,\bar{A}_2 + \text{Acidic caseinogen}.$$

In the following pages evidence is brought forward in favour of the view that the main protein of milk consists of a complex of protein and calcium phosphate in definite proportions, and that the protein precipitated from milk by the addition of acid (acidic caseinogen) is different from that contained in the caseinogen complex. My conclusions are at variance with those

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of other workers in so far as they have assumed that acidic caseinogen is essentially the same as caseinogen and have used the former protein as the basis of their experimental work. I propose to defer the consideration of the properties of caseinogen and the relation of acidic caseinogen to it.

The preparation of caseinogen from milk. In a previous paper [1907] I have shown that the precipitation of a protein from solution by alcohol usually involves two distinct processes; (i) precipitation of the protein; (ii) coagulation of the protein, this latter process rendering the precipitate insoluble in the original solvent. In the case of the protein of horse serum these two processes can be readily dissociated by precipitation above or below 14° C. Below this temperature, alcohol, added to the extent of 60 %, precipitates the protein in an unchanged condition; above this temperature the protein is not only precipitated but also rendered insoluble, the degree of insolubility of the precipitated protein being a function of (*a*) the temperature of precipitation, (*b*) the amount of alcohol used and (*c*) the length of time during which the precipitated protein is left in contact with the alcohol. These actions of alcohol are dependent upon the concentration of electrolytes contained in the protein solution, the less the ionic energy per unit volume the more effective the precipitating action of a definite quantity of alcohol, and the less insoluble the resulting precipitated protein. Similar facts hold good for the protein of milk. If alcohol, to the extent of 50 %, be added to fresh milk, caseinogen is not only precipitated but also coagulated. If, however, alcohol be added to the extent of 75 % to milk cooled below 15° C., the resultant protein precipitate, when freed from alcohol, is readily soluble in water.

The following method was used for the precipitation of caseinogen from milk by alcohol.

To one volume of separated milk three volumes of alcohol were added, both the milk and the alcohol being cooled below 15° C. The precipitate, after being strained through muslin and so freed from excess of alcohol, was ground up in a mortar with ether, filtered, and again extracted with ether until freed from fat and pigment. The final product was pressed between folds of filter paper and allowed to dry in the air. It is convenient to use fresh separated milk since milk fat accompanies caseinogen in the alcohol precipitation, and a large quantity of fat requires a large quantity of ether for its extraction. Also it is not advisable to cool the alcohol to 0° C. since at this temperature a large quantity of lactose is precipitated with the caseinogen.

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The precipitate obtained from milk by alcohol consists of caseinogen admixed with small quantities of lactose, lactalbumin, and inorganic salts. The solid is not dry in the sense that it does not lose weight when heated to 100° C. It is advisable to keep it in an impure condition in a stoppered bottle since if heated to 100° C. in an air bath it passes into a modification insoluble in water.

The composition of caseinogen. The composition of caseinogen was determined by making a series of experiments on the impure substance. The caseinogen was not separated from all impurities before determining its properties since such a procedure involves prolonged manipulation, and caseinogen being a labile protein readily passes into modifications which differ from the original substance. The crude substance when suspended in water at 37° C. forms a milk white solution. This solution takes place slowly (about thirty minutes) probably owing to the difficulty of the water getting into intimate contact with the large protein aggregates. An important fact to notice, however, is that the crude substance contains no substances insoluble in water such as calcium phosphate, or a protein comparable in solubility to acidic caseinogen.

The impurities associated with caseinogen in the crude alcoholic precipitate can be estimated by taking advantage of their solubilities compared with that of caseinogen. Caseinogen is practically insoluble in water at 15° C., whilst lactose and lactalbumin are freely soluble at this temperature. Therefore in order to determine the amount of caseinogen contained in five grams of the solid, this quantity of substance was suspended in water, made up to 100 cc., and allowed to extract at room temperature (15° C.) for twenty hours. At the end of this time the suspension was filtered, and the weight of solid in a measured quantity of the filtrate determined by drying over a water bath and completing the drying in an air bath at 120° C. for four hours.

The following figures give the results of such an analysis for one gram of the impure solid:

Weight of substance soluble in water at 15° C. contained in 20 cc. of filtrate	0·19 g.
Loss in weight of 1 g. of solid after drying at 120° C. for four hours (H_2O, etc.) ...	0·202 g.
	0·392 g.

Therefore one gram of the solid contained 0·608 g. of caseinogen.

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(*a*) *The calcium and phosphorus content of caseinogen.* The amount of calcium contained in caseinogen was determined by the method of Hurtley and Kahn [1915]. The method consists of ashing a known weight of caseinogen, dissolving the ash in a small quantity (5 cc.) of 25 % phosphoric acid, diluting to 100 cc. with water, precipitating the calcium by slowly adding from a burette 100 cc. of 5·524 % potassium oxalate, washing the precipitate with water till free from soluble oxalate, dissolving it in dilute sulphuric acid, and determining the quantity of freed oxalic acid (and consequently calcium) by titration with tenth normal permanganate. The following figures give the results of a typical experiment made in this way.

Calcium contained in one gram impure, undried caseinogen

$$= 12{\cdot}8 \text{ cc. } KMnO_4 \text{ (0·1 N)}.$$

The calcium contained in the substances soluble in water at 15° C. = 3·08 cc. $KMnO_4$ (0·1 N).

From the figures previously given the weight of caseinogen contained in one gram of impure substance = 0·608 g.

Therefore the amount of calcium contained in one gram of pure caseinogen = 15·84 cc. $KMnO_4$ (0·1 N) = 0·0317 g. calcium.

The amount of phosphorus contained in the caseinogen was determined by a modified Neumann's [1902] method. Amounts of impure caseinogen varying in weight from 0·2 g. to 0·5 g. were oxidised by the method described by Plimmer and Bayliss [1906]. In this oxidation it was necessary to use small quantities of the protein and prolonged heating with nitric acid to ensure complete destruction of the protein. The resultant fluid was diluted to 100 cc. and after heating to 80° C., 100 cc. of a phosphate precipitating mixture was added, and the heating continued on a water bath for 15 minutes. For precipitation of the phosphate a filtered mixture of ammonium nitrate and ammonium molybdate in nitric acid made up in the proportions recommended by Halliburton [1914] was used.

This solution does not deposit molybdic acid in the cold but when heated to 100° C. the white modification of molybdic acid separates out. The precipitation of molybdic acid is prevented by sulphuric acid, and it was essential therefore so to conduct the preliminary hydrolysis of the protein that sufficient sulphuric acid remained in the hydrolysed fluid to prevent the precipitation of molybdic acid, but not that of the ammonium phosphomolybdate, after the addition of the precipitating mixture, and subsequent heating on a water bath. The following gives the experimental

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results obtained from the same sample of crude caseinogen as was used in the experiments above.

P_2O_5 in one gram impure caseinogen = 38·5 cc. NaOH (0·5 N).

P_2O_5 contained in the substances soluble in H_2O at 15° C. = 9 cc. NaOH (0·5 N).

Therefore the P_2O_5 contained in the caseinogen was equivalent to 29·5 cc. NaOH (0·5 N).

The weight of caseinogen contained in one gram of impure substance = 0·608 g.

Therefore the amount of P_2O_5 contained in one gram of pure caseinogen = 48·6 cc. NaOH (0·5 N) = 0·062 g. P_2O_5.

(*b*) *The calcium and phosphorus contents of acidic caseinogen.* On the addition of acid to a solution of caseinogen a protein (acidic caseinogen) is precipitated. A comparison of the calcium and phosphorus contents of caseinogen and acidic caseinogen yields interesting results. The acidic caseinogen used in these experiments was prepared by dissolving the impure caseinogen in water, precipitating the acidic caseinogen by the addition of an optimal quantity of acetic acid and washing the precipitate with water, alcohol and ether. It may be remarked that acidic caseinogen prepared in this way is identical in phosphorus content with that prepared by the acidic precipitation of fresh milk, and with that of Merck's "pure casein" (acidic caseinogen) prepared by Hammarsten's method. The identity of the results obtained with these specimens of acidic caseinogen affords presumptive evidence in favour of the view that caseinogen precipitated from milk by alcohol is the protein of milk in an unaltered condition.

Acidic caseinogen prepared from caseinogen contains no calcium and in this respect also agrees with the analysis of acidic caseinogen obtained from milk by Hammarsten's method. The absence of calcium is in marked contrast to the large quantities of this element contained in caseinogen.

The phosphorus content, however, although less than that of caseinogen, is large. The following figures give the average of a series of experiments.

P_2O_5 in one gram acidic caseinogen (pure) = 16·5 cc. NaOH (0·5 N) = 0·021 g. P_2O_5.

(*c*) *A comparison of the calcium and phosphorus contents of caseinogen and acidic caseinogen.* From the figures given above it may be seen that one gram of caseinogen contains 0·0317 g. calcium and 0·062 g. P_2O_5, and one gram of acidic caseinogen contains no calcium and 0·021 g. of P_2O_5.

The question arises as to whether these figures give any indication of

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the relation of caseinogen to acidic caseinogen, and the manner in which calcium is contained in the caseinogen complex. We may legitimately assume that the phosphorus contained in acidic caseinogen is an organic combination since there are no inorganic bases to combine with it. If this amount of phosphorus be organically contained in caseinogen also, there is now 0·0406 g. P_2O_5 contained in one gram of caseinogen not accounted for. On the assumption that the calcium is associated with the residual P_2O_5 (0·0406 g.) in the caseinogen complex as $Ca_3(PO_4)_2$, 0·034 g. of calcium would be required. The approximation of the theoretical quantity of calcium required (0·034 g.) with that actually determined (0·0317 g.) offers strong evidence in support of the hypothesis that in caseinogen the phosphorus is present in two forms (*a*) in organic combination, (*b*) associated with calcium as calcium phosphate.

(*d*) *A comparison of the quantity of phosphorus contained in acidic caseinogen with that of inorganic phosphorus contained in caseinogen.* The amount of phosphorus contained in one gram of acidic caseinogen is 0·021 g. P_2O_5. On the assumption that a molecule of acidic caseinogen contains at least one atom of phosphorus, the least molecular weight for caseinogen is 3375, i.e. 3375 g. of acidic caseinogen contain one gram atom of phosphorus.

The amount of inorganic phosphorus assumed to be present in one gram of caseinogen is 0·0406 g. P_2O_5, the assumption being that this phosphorus is present in the protein complex associated with calcium in the same proportions as in calcium phosphate. From these figures it may be calculated that 142 g. of P_2O_5 (the quantity contained in a gram molecule of calcium phosphate) are contained in 3480 g. of caseinogen. The remarkable approximation of the two numbers giving (1) the least molecular weight of acidic caseinogen on the assumption that an atom of phosphorus is contained in the molecule (3375) with (2) the quantity of caseinogen complex which contains a gram molecule of calcium phosphate (3480 g.) affords strong evidence in favour of the assumption made above, i.e. that caseinogen contains phosphorus in two forms (*a*) organically combined in the protein molecule, identical with the phosphorus in acidic caseinogen (0·021 g. P_2O_5 per gram of caseinogen) and (*b*) contained in caseinogen complex in association with calcium as $Ca_3(PO_4)_2$ (0·406 g. P_2O_5 per gram of caseinogen). This hypothesis receives further confirmation from a consideration of the quantity of acetic acid required to precipitate acidic caseinogen from caseinogen.

(*e*) *The precipitation of caseinogen by acetic acid.* On the addition of acid to a solution of caseinogen no precipitate is produced until a definite

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quantity of acid has been added and then the whole of the acidic caseinogen comes out of solution. The following figures illustrate this statement.

Caseinogen solution	H_2O	Acetic acid (0·1 N)	
1 cc.	8·9 cc.	0·1 cc.	no pp.
	8·8 ,,	0·2 ,,	,,
	8·7 ,,	0·3 ,,	,,
	8·6 ,,	0·4 ,,	,,
	8·575 cc.	0·425 cc.	trace pp.
	8·55 ,,	0·45 ,,	complete pp.

This precipitation will be considered more fully later. At present it may be noted that a definite quantity of acid precipitates a definite quantity of caseinogen, and that these relations are almost independent of temperature or dilution. In the above experiment the quantity of caseinogen contained in the solution was 2·68 g. per 100 cc. Therefore 0·45 cc. acetic acid (0·1 N) precipitated 0·0268 g. caseinogen, or in ordinary terminology, a gram molecule of acetic acid precipitated 583 g. of caseinogen from solution as acidic caseinogen. Now six molecules of acetic acid are equivalent to one molecule of calcium phosphate $Ca_3(PO_4)_2$. Therefore an amount of acetic acid which is equivalent to a gram molecule of calcium phosphate precipitates 3498 g. of caseinogen from solution as acidic caseinogen. The interest of this number lies in the fact that the quantity of caseinogen which contains a gram molecule of calcium phosphate is 3480 g.

Discussion of results.

The above results may be stated shortly as follows:

I. On the assumption that one unit of acidic caseinogen contains one atom of phosphorus the weight of acidic caseinogen containing one gram atom of phosphorus is 3375 g.

II. On the additional assumption that the phosphorus of caseinogen is contained in two forms (i) as organic phosphorus equal in quantity to that contained in acidic caseinogen, (ii) as inorganic phosphorus being contained in the protein complex as calcium phosphate we find (*a*) that the quantity of calcium and inorganic phosphorus contained in caseinogen bears the same relation to one another as is expressed in the formula $Ca_3(PO_4)_2$ and (*b*) that the amount of caseinogen which contains one gram molecule of calcium phosphate is 3480 g.

III. The amount of caseinogen precipitated by six gram molecules of acetic acid, i.e. a quantity equivalent to a gram molecule of calcium phosphate, is 3489 g.

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The approximation of the above numbers—the quantity of acidic caseinogen containing a gram atom of phosphorus (3375 g.); the quantity of caseinogen containing a gram molecule of calcium phosphate (3480 g.); and the quantity of caseinogen precipitated by six gram molecules of acetic acid (3489 g.)—offers strong evidence in favour of the assumptions made and justifies the conclusions that,

(1) a unit of acidic caseinogen containing one atom of phosphorus has a weight of approximately 3500;

(2) caseinogen consists of a complex of protein having the same weight as the acidic caseinogen unit associated with one molecule of calcium phosphate;

(3) the precipitation of acidic caseinogen from caseinogen by acetic acid involves the reaction of the added acid with equivalent quantities of calcium phosphate, one molecule of calcium phosphate being replaced by six molecules of acetic acid.

The following tentative hypothesis is put forward as to the constitution of the caseinogen complex.

The protein contained in the complex may be expressed by the formula

$$R\begin{cases}(NH_2)_2\\(X)\end{cases}$$

of which the radicle (X) has acidic properties, possibly due to the phosphorus contained in it. The acidic radicle (X) reacts with the $Ca_3(PO_4)_2$, forming X Ca, whilst the liberated acid calcium phosphate combines with the amino group, the resultant complex having the formula

$$R\begin{cases}(NH_2)_2\,Ca_2H_2(PO_4)_2\\X\ Ca\end{cases}$$

The experimental evidence in favour of this decomposition of $Ca_3(PO_4)_2$ into $Ca(OH)_2$ and $Ca_2H_2(PO_4)_2$ is derived from the work of Cameron and Seidell [1904] who showed that tricalcium phosphate and monocalcium phosphate are considerably decomposed by water but that dicalcium phosphate is relatively stable in this medium.

Summary.

(1) Caseinogen may be prepared from milk by alcohol precipitation. The protein forms a milk white solution in water possessing all the properties of caseinogen contained in milk.

(2) Comparative analyses of caseinogen and acidic caseinogen indicate that caseinogen is composed of a complex of protein and calcium phosphate,

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approximately 3500 g. of caseinogen containing a gram molecular weight of tricalcium phosphate.

(3) The amount of phosphorus contained in acidic caseinogen shows that a gram atom of phosphorus is contained in approximately 3400 g. of acidic caseinogen.

(4) Six gram molecules of acetic acid (a quantity equivalent to one gram molecule of calcium phosphate) precipitate acidic caseinogen from approximately 3500 g. of caseinogen.

(5) These relations suggest that the protein unit of acidic caseinogen has a weight of approximately 3400 g.; that caseinogen consists of a complex containing one unit of protein and a molecule of tricalcium phosphate; and that the precipitation of acidic caseinogen from caseinogen by acetic acid may be expressed thus:

$$\underset{\text{(caseinogen)}}{\text{Protein, } Ca_3(PO_4)_2} + 6\,H\bar{A} = \underset{\text{(acidic caseinogen)}}{\text{Protein, } 6\,H\bar{A}} + Ca_3(PO_4)_2$$

The following relation of the protein to the calcium phosphate in caseinogen has been suggested:

$$R\begin{cases}(NH_2)_2\,Ca_2H_2\,(PO_4)_2 \\ X\ Ca\end{cases}$$

where the group (X) has feeble acidic properties.

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XXXVI. THE COMPOSITION OF "LECITHIN," TOGETHER WITH OBSERVATIONS ON THE DISTRIBUTION OF PHOSPHATIDES IN THE TISSUES AND METHODS FOR THEIR EXTRACTION AND PURIFICATION.

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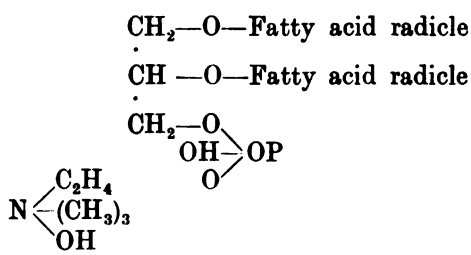
(*Received August 17th, 1915.*)

About the year 1846 Gobley succeeded in obtaining from egg yolk and other organs a substance of a fat-like nature containing nitrogen and phosphorus which he called lecithin [1846]. This substance was soluble in alcohol and in ether, formed an emulsion when rubbed up with water and yielded fatty acids and glycerophosphoric acid on hydrolysis. These observations of Gobley furnished a basis for the structure of the lecithin molecule, which appeared to be completed by the discovery of Liebreich [1865] and Strecker [1868] that the base choline was also obtained as a product of lecithin hydrolysis.

Lecithin was therefore defined as an ester compound of glycerophosphoric acid substituted by two fatty acid radicles with a base, choline, according to the following formula which is generally accepted.



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Later it was found that other somewhat similar bodies were present in different organs and tissues; for this general class of substances the name *phosphatides* was suggested by Thudichum [1884].

Lecithin is soluble in all the ordinary organic solvents with the exception of acetone. It differs from a substance known as kephalin in that the latter is insoluble in alcohol. So far, as has already been pointed out, the only phosphatides identified with certainty as distinct substances are lecithin, kephalin, cuorin and sphingomyelin.

A short description of these substances and their probable distribution in the tissues will help to render clear certain problems discussed later.

Both kephalin and lecithin are monamino-monophosphatides (N : P = 1 : 1). Cuorin on the other hand is a monamino-diphosphatide (N : P = 1 : 2), while sphingomyelin is a diamino-monophosphatide (N : P = 2 : 1). The first three of these substances are more or less of a plastic consistency and very hygroscopic, while they are so labile that exposure to air and light for a comparatively short time considerably modifies certain of their properties. They do not crystallise and on account of their liability to oxidation must be preserved in evacuated desiccators. Sphingomyelin on the other hand is a pure white crystalline substance and has physically no resemblance to the other phosphatides mentioned. It is practically non-hygroscopic and does not oxidise on exposure to air.

While these substances have many properties in common, their separation and identification depend chiefly on the following points.

	Lecithin	Kephalin	Cuorin	Sphingomyelin
(1)	Soluble in ether and in alcohol	Sol. in ether Insol. in alcohol	Sol. in ether Insol. in alcohol	Insol. in ether Sol. in hot alcohol Insol. in cold alcohol
(2)	Insol. in acetone	Insol. in acetone	Insol. in acetone	Insol. in acetone
(3)	N : P = 1 : 1	N : P = 1 : 1	N : P = 1 : 2	N : P = 2 : 1

The two chief solvents in common use for extracting phosphatides from tissues are ether and alcohol. Ether extracts all four phosphatides to some extent, for although sphingomyelin is insoluble in ether it is fairly soluble in an ether extract of tissue. On the other hand, only a small part of the total tissue phosphatides are capable of being extracted by means of ether. After extracting with ether to a point at which only the merest traces of phosphatides can be obtained, it is found that subsequent extraction with alcohol results in the removal of further large amounts of these substances.

Attempts have been made to explain this phenomenon on the supposition that the ether fails to penetrate the tissues—that the difficulty is simply

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a mechanical one. It has been pointed out that when fats or phosphatides are mixed with protein matter and the whole mass dried and then extracted with ether, only a fraction of the original ether-soluble material is removed. While this is true to a degree, it is equally certain that the fraction which can be removed bears a much higher proportion to the total fatty matter present than is the case in extraction of the tissues.

In extracting dried normal kidneys with ether not more than 15 % to 30 % of the total phosphatides can generally be obtained; often the amount is even less than this. On subsequent extraction with alcohol the remainder comes out with ease.

If, however, the kidney is one showing marked fatty degeneration, the greater part of the phosphatides is obtained by ether extraction.

From this it would appear that part of the phosphatide material at any rate is present in some kind of ether-insoluble combination with the proteins; this combination is acted on by alcohol and the phosphatide liberated; it is then easily extracted by ether in so far as physical difficulties permit. In this connexion an interesting observation has just been made by Osborne and Wakeman [1915]. In an investigation on milk they found that the phosphatides were present in the precipitated protein matter; on drying this protein and extracting it with ether no lecithin whatever was obtained, while subsequent treatment with alcohol removed a considerable amount. The explanation advanced by Erlandsen [1907] that the ether-extractable material was "lecithin" while the ether-soluble substance subsequently obtained by means of alcohol was not "lecithin" but a new substance—a diamino-monophosphatide—has been shown to be untenable [MacLean, 1913, 1].

By far the greater part of the tissue phosphatides generally consists of the so-called "lecithin" together with some kephalin. Cuorin appears to be present only in small amount, and from many tissues only a trace of sphingomyelin can be obtained.

On an alcohol-soluble nitrogenous impurity associated with "lecithin."

The observations already made show that alcohol is a more suitable solvent than ether for extracting lecithin from tissues. For the reasons given, alcohol is now generally used, the usual procedure being to dry the tissue as quickly as possible, grind it to a fine powder and extract thoroughly with various changes of the solvent, a shaking machine being used. On evaporating the

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alcohol from the combined extracts the residue is taken up with ether, and the phosphatides precipitated by the addition of excess of acetone to the ethereal solution. By repeating the process several times all acetone-soluble bodies such as cholesterol and fatty acids are removed. The final residue is supposed to consist almost entirely of phosphatides and this is divided into "lecithin" and kephalin by fractionation with alcohol. The alcohol-insoluble part is considered as kephalin; the alcohol-soluble as "lecithin."

In every case, however, in which this method is used as a basis for the preparation of phosphatides, the resulting products are far from pure and the "lecithin" fraction often contains as much as 50 % of an extraneous product. This substance which has already been referred to [MacLean, 1912] is soluble in water and on treatment of the "lecithin" fraction by means of acetone and water [MacLean, 1912] it remains in the acetone-water solution. On evaporation of this solution the substance is obtained as a sticky syrupy mass which is insoluble in ether and in *absolute* alcohol but easily soluble in alcohol containing a trace of water. It is exceedingly soluble in water; on standing for some days an aqueous solution gradually deposits small round white crystals. These crystals are insoluble in cold water, but dissolve in boiling water to be reprecipitated on cooling. After recrystallising in this way several times, a substance or mixture of substances is obtained which has a very high nitrogen content and evidently belongs to the purine group.

In one case small round concentric white crystals were isolated which had all the properties of carnine. After drying at 105°, analysis showed that the substance contained 28·55 % nitrogen. On heating it changed colour about 230° and blackened at 240°. With silver nitrate a white flocculent precipitate was obtained which did not dissolve in ammonia or nitric acid. A precipitate was also given with basic lead acetate and with mercuric chloride, while neither neutral lead acetate nor mercuric nitrate gave any result. In its properties and nitrogen content this substance appears to be identical with or closely related to the base carnine found by Weidel in American meat extract. In another sample a small amount of a substance which appeared to be impure hypoxanthine was isolated; it contained about 40 % nitrogen. The mother liquor from which these substances separate is distinctly acid in reaction, and it is probable that these bodies are set free by a process of decomposition and are not present in the free state in the original liquid. This is suggested by their extreme insolubility in cold water and the difficulty experienced in redissolving them in the

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mother liquor after separation has once taken place. The fact that complete separation takes days or even weeks is also suggestive in this connexion.

Owing to the high nitrogen percentage in these bodies it is obvious that a small quantity present in lecithin would materially influence the N : P ratio.

When the separation of these bodies is complete an absolutely clear straw coloured mother liquor is obtained which remains clear for a very long time.

On evaporating, a sticky hard gum-like substance remains. This substance is soluble in alcohol containing a trace of water and appears to dissolve in solutions of lecithin. Further, like phosphatides in general, *it is precipitated by acetone, by cadmium chloride, and also by platinum chloride.* The body appears to be of a very complex nature and so far it has been impossible to determine its chemical constitution. Different specimens contain on an average about 6 % of nitrogen, and there is generally a small amount of phosphorus also present, though the latter may not amount to more than a trace. When tested by Van Slyke's method a considerable part of its nitrogen was found to be present in the amino form. On hydrolysis some specimens yielded a very small amount of fatty acids, but this was probably due to contamination with lecithin, for a specimen has been obtained recently which, on hydrolysis with HCl, gave no trace of fatty acids. It decolourised Fehling's solution on heating but no precipitate occurred: on prolonging the boiling however a dense precipitate was formed.

Attempts to fractionate the water-soluble substance.

Some of the substance was rubbed up thoroughly with ether to remove any traces of fatty acids or phosphatides that might be present. The ether was decanted off and the residue dissolved in about 20 cc. water to which 30 cc. alcohol and 50 cc. ether were added. On the addition of the ether, part of the substance was precipitated as an oily liquid. This was dried and analysed. It contained 5·7 % nitrogen and 0·9 % phosphorus.

The part that remained soluble in the water-alcohol-ether mixture was obtained by evaporating off the solvent. It was of a brown colour and thick syrupy consistency. This was dissolved in water and excess of mercuric chloride added which produced an immediate precipitate.

After standing for two hours this precipitate was filtered off = *Precipitate A*. The filtrate was allowed to stand, and gradually deposited some more substance

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which was filtered off after 24 hours = *Precipitate B*. Precipitate *A* was decomposed in water by means of sulphuretted hydrogen, the insoluble sulphide separated and the filtrate evaporated to dryness *in vacuo*. A crop of crystals was obtained; the nitrogen estimation gave the following figures:

0·0164 g. required 4 cc. N/10 H_2SO_4 = 34·1 % N.

In certain of its properties it agreed with creatinine, though on treatment with sodium nitroprusside and alkali, or picric acid and alkali, no red colour was produced. Sufficient of the substance was not obtained to identify it with certainty; it was probably not a pure substance. Precipitate *B* was treated in the same way as above, and a small amount of crystalline residue was obtained. Unlike the corresponding substance obtained from precipitate *A*, this substance gave marked creatinine reactions and presumably contained creatinine which might have been formed from creatine during the various manipulations employed.

Thus far there is evidence that the original water-soluble impurity contained carnine or somewhat similar bases, and a trace of creatinine, the latter perhaps being an artificial product. The filtrate *M* from the mercuric chloride precipitate was decomposed by sulphuretted hydrogen and the sulphide separated by filtration. The filtrate was evaporated to dryness on the water bath and the residue dissolved in alcohol. After filtration the alcohol was removed *in vacuo* and the residue dried and analysed for N and P. It was a brownish hard hygroscopic material.

0·0708 g. required 3·09 cc. N/10 H_2SO_4 = 6·11 % N.
0·2234 g. gave no trace of phosphorus.

These results sufficiently indicate the complex nature of this substance; the fact that it is so exceedingly difficult to separate from the phosphatides by the ordinary methods of purification is undoubtedly responsible for the appearance in the literature of many supposed new phosphatides.

In very many cases it is quite obvious from the methods employed by several investigators that the phosphatides obtained and supposed to be pure substances must have been contaminated with this impurity.

The phosphatides can be entirely separated from this impurity by emulsification with water and precipitation with acetone as already described in a former paper [MacLean, 1912].

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Some anomalous results obtained in the extraction and preparation of phosphatides.

The simplest method for the extraction of the chief bulk of the phosphatides from the tissues is by means of alcohol, the dried tissue being thoroughly exhausted with this solvent. If the cuorin is also wanted it is necessary to use ether, as this substance is apparently quite insoluble in alcohol or in alcoholic solutions of phosphatides. When the alcohol filtrates are concentrated, the residue dissolved in ether and the phosphatides precipitated by excess of acetone, it is generally found that a good deal of the acetone precipitate is insoluble in absolute alcohol. This insoluble part on further purification is found to have a N : P ratio of 1 : 1 and constitutes the substance known as kephalin; the alcohol-soluble "lecithin" generally predominates in amount. If, however, the same kind of tissue is obtained in as fresh a condition as possible and carefully and quickly dried, either by immersing the finely divided substance for a few minutes in excess of alcohol or by exposing it in thin films to a current of air at 30°, it is often found that the alcoholic extract, when treated in the usual way with acetone, gives a precipitate which is entirely soluble in alcohol. In this case the whole of the extracted phosphatide, since it is alcohol-soluble, would be held to be "lecithin." Now these divergent results can be obtained from the same tissue, so that according to the treatment of the material one observer may find nothing but "lecithin" while another may find abundance of both "lecithin" and kephalin.

Again, it very often happens that "lecithin," which dissolves easily in alcohol immediately after its separation from the tissues, becomes partly alcohol-insoluble even when preserved in a carefully evacuated brown glass desiccator. The alcohol-insoluble substance formed has the properties and general composition of kephalin. This change in solubility is much more in evidence if the organic solvents used are not quite pure, and in general the greater care taken to purify the various organic reagents—ether, acetone, etc.—the more "lecithin" is obtained. Particularly does this appear to be the case with ether.

In one experiment bearing on this point heart tissue was extracted with alcohol in the ordinary way: the residue was taken up in ordinary "good" ether as supplied by the dealers. Another portion was treated in exactly the same way, ether from the same source being used, but only after careful purification. Using ordinary ether a considerable amount of the phosphatide

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ultimately obtained was insoluble in alcohol and part of the "lecithin" fraction became alcohol-insoluble after a week; on the other hand the specimen in which purified ether was used gave nearly all "lecithin" which, up to the present time (six weeks after preparation), remains quite soluble in alcohol.

From these anomalous results it is easy to explain the divergence of opinion with regard to the occurrence of kephalin in the tissues.

Thus, Erlandsen in heart muscle could obtain no trace of kephalin [1907], while Koch and Woods [1905] found kephalin in the heart tissue in excess of the lecithin in the proportion of 100 to 61. In many other cases these observers found more kephalin than lecithin as the following figures show.

Substance analysed	Total phosphatide per cent.	Lecithin per cent.	Kephalin per cent.
Egg yolk	9·9	3	6·9
Pancreas	1·89	0·59	1·3
Milk (cow)	0·086	0·049	0·037
Liver	2·3	0·8	1·5
Kidney	2·6	1·2	1·4

How are we to explain these results? The explanation appears to be that kephalin, when rapidly extracted from fresh tissues, is soluble in an alcoholic solution of "lecithin." Its insolubility is probably brought about by oxidation, so that when impure solvents are used or precautions to prevent chemical changes are not taken, some of the kephalin becomes insoluble. If these oxidation changes are very marked some of the lecithin may also become insoluble so that the "kephalin" represents in this case a mixture of true lecithin and kephalin; it would appear however that true lecithin is much less liable than kephalin to become alcohol-insoluble.

A good deal of controversy exists with regard to the question whether kephalin is absolutely or relatively insoluble in alcohol. It is obvious, however, that kephalin is not entirely alcohol-insoluble, more especially in the presence of other phosphatides. If, therefore, a tissue is treated with alcohol, two monamino-monophosphatides are extracted—lecithin and kephalin—but it depends on circumstances whether the kephalin is insoluble in the alcohol or remains dissolved in the alcoholic solution of lecithin. In the latter case the phosphatide would have all the general properties of "lecithin" and little or no kephalin would be obtained. The small amount of sphingomyelin present in the tissues would also be present in the alcohol extract.

From this it appears that the substance known as "lecithin" must in many, if not in all cases, be represented by a mixture of two substances—

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kephalin which tends to become insoluble in alcohol, and a substance which remains alcohol-soluble—true lecithin. The lecithin of the text books is really a lecithin-kephalin mixture. While it is possible that the two substances may be present in some kind of feeble combination there is obviously no combination in the ordinary chemical sense; this is indicated by results to be described later. For the further discussion of the subject it is necessary to point out the chief differences which have been established between kephalin and true lecithin.

Kephalin. Kephalin owing to its insolubility in alcohol and the conditions just described can be obtained in the pure state. The pure substance seems to have the same general structure as true lecithin, but in the case of kephalin the base present is not choline but β-amino-ethyl alcohol. This base is difficult to isolate in that its combination with platinum chloride is soluble in alcohol, especially in the presence of glycerol or other products of phosphatide hydrolysis. The salt is precipitated from its alcoholic solution by ether. It may, however, be obtained as the platinum chloride or gold chloride double salt by careful concentration. It yields all its nitrogen when treated with nitrous acid and can be estimated quantitatively by Van Slyke's method. Its compounds are so soluble that a gravimetric method of estimation has not yet been found.

Kephalin is precipitated by cadmium chloride in alcohol solution, the *resulting combination being soluble in ether.*

True lecithin. Unlike kephalin, true lecithin has theoretically all its nitrogen in the form of choline. Hitherto it is probable that no one using the ordinary methods has succeeded in obtaining a lecithin in which the choline accounted for the whole of the nitrogen. That the "theoretical" compound does exist will be shown later. Like kephalin, lecithin is precipitated by cadmium chloride, but *under certain circumstances the precipitate is insoluble in ether.*

THE COMPOSITION OF ORDINARY "LECITHIN."

The above observation, that ordinary lecithin is a mixture, is strengthened by the results of many investigators who found that the amount of choline obtainable from lecithin, and generally separated as the platinum compound, was not nearly sufficient to account for the whole of the nitrogen present. The figures given indicate sufficiently the variations found.

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Investigator	Source of "lecithin"	Percentage of nitrogen of "lecithin" obtained as choline platinum chloride
Heffter [1891]	Liver	25
Erlandsen [1907]	Heart	42
Moruzzi [1908]	Egg yolk	77
MacLean	Heart	42 to 75
"	Egg yolk	65
"	Other specimen egg yolk	77
"	Trade lecithin (Riedel)	80
"	Trade lecithin (Kahlbaum)	90
Osborne and Wakeman [1915]	Milk	39·5

It is exceedingly suggestive that "lecithins" from the same source but prepared in different ways did not always contain the same percentage of choline, while control experiments with the same sample gave almost identical results. As a result of similar experiments the writer many years ago came to the conclusion that "the generally accepted lecithin formula in which the whole of the nitrogen was represented by a base—choline—is incorrect and can no longer be accepted" [1909, 1]. The correctness of this view was questioned by Malengreau and Prigent [1912] who, working with Kahlbaum's lecithin, obtained nearly the whole of the nitrogen as choline. They made use of the cadmium chloride salt of lecithin and hydrolysed for long periods by means of N/10 H_2SO_4. That certain trade lecithins do contain a large percentage of their nitrogen as choline had been already emphasised by me [1908], but in cases where I employed acid hydrolysis the acid used was much stronger than the above. I therefore thought it well to repeat the experiment of Malengreau and Prigent with "lecithins" prepared from heart and from eggs.

Method of separation of choline from lecithin.

The lecithin was boiled for several hours (14 to 72) with excess of N/10 H_2SO_4; the mixture was then allowed to cool, when the fatty acids separated out as a solid cake on the surface. This was separated by filtration. The fatty acids were then returned to the flask, some distilled water added and the whole boiled for several minutes. On cooling the acids which again separated as a solid cake were as before removed by filtration. This process was carried out from four to six times.

An aliquot part of the total liquor obtained was taken and neutralised with $Ba(OH)_2$ solution; the precipitate formed was separated by filtration and carefully washed many times with boiling water. The filtrate, acidified with hydrochloric acid, was evaporated to dryness on the water bath, the residue extracted with alcohol, the alcoholic solution filtered, made up

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to a definite volume and divided into two equal parts. One part was used for estimation of the nitrogen, and the other for choline platinum chloride. The results given below were very similar to those obtained in my earlier experiments with stronger acids.

Source of lecithin	Time of hydrolysis in hours	Percentage of nitrogen of final filtrate obtained as choline platinum chloride
Kahlbaum's trade lecithin	14	91·4
„ „	35½	92
Heart (old specimen)	16	89
„ (by ether extraction)	32	63·8
„ (by subsequent alcohol extraction)	32	69
„ (direct alcohol extraction) ...	35½	72
„ (ether extraction)	35½	61·4
„ „ „ (control) ...	35½	62
„ (ether extraction, very fresh specimen)	30	52

The difference in choline content of "lecithins" from the same source is well brought out and is important in considering whether the "lecithin" is a mixture of true lecithin and kephalin or a combination. These results suggest that it is merely a mixture and not a combination, for in the latter case it is difficult to see how this variation in nitrogen content could arise.

Further evidence in support of this contention is furnished by an observation made several years ago [MacLean, 1909, 2] that when "lecithin" is precipitated by cadmium chloride, the cadmium compound obtained contains considerably more of its nitrogen as choline (in one case 10 % more) than the lecithin from which it was obtained. This observation has been confirmed by Eppler [1913] and also by Trier.

In my experiments, the cadmium chloride precipitate was washed with ether and it is possible that this, rather than the alcohol, was the cause of the difference. This, however, does not in any way influence the deduction to be drawn—that we are dealing with a mixture and not a compound.

There still remained the theoretical possibility that none of the methods used to estimate choline was satisfactory, and that all the nitrogen might still be present as choline.

These objections were answered by the discovery of Trier [1911], who found β-amino-ethyl alcohol $CH_2(NH_2).CH_2.OH$ among the products of hydrolysis of the phosphatides of bean meal (*Phaseolus vulgaris*). Later the same observer recovered amino-ethyl alcohol from egg lecithin, identifying it by means of the gold chloride combination [1912, 1, 1913]. Trier has shown that amino-ethyl alcohol when methylated with methyl iodide, methyl alcohol and potassium hydroxide is converted into choline [1912, 2].

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Amino-ethyl alcohol was also discovered independently in egg lecithin by Eppler [1913] working in Thierfelder's Laboratory, and has been found several times by the writer in heart "lecithin" [MacLean, 1913, 2]. It has also been found by Trier in various other "lecithins" particularly of plant origin. The next point to determine was whether the whole of the nitrogen of "lecithin" can be accounted for by the choline and amino nitrogen present. The amino nitrogen may be assumed to be present as amino-ethyl alcohol, since there is abundant evidence that this base is always present in "lecithin"; this view is supported by the fact that no other base containing amino nitrogen can be obtained from lecithin. There are also many chemical considerations such as the extreme solubility of the platinum and gold chloride combinations of this substance which are suggestive. In hydrolysed lecithin, platinum chloride precipitates only choline while another combination with a base remains in solution in the filtrate. On carefully evaporating this filtrate, the only base obtained is the amino-ethyl alcohol salt which appears to be invariably present. Before giving details of the experiments performed to determine the distribution of nitrogen in "lecithin" the method by which the "lecithin" was prepared will be described, as this differs considerably from the ordinary methods and has been somewhat modified of late. The "lecithin" used in all the experiments to be described later, together with several "lecithins" already mentioned, was prepared in this way. The following are the chief steps, which may be somewhat varied according to circumstances.

Preparation of "Lecithin."

(1) The tissue used (heart, kidneys or egg yolk) was in the case of solid organs ground to a fine paste by a mincing machine, the paste spread out in a thin layer on a glass plate and dried at 30° by means of an air current generated by a fan. Sometimes the original paste was put into excess of alcohol for a few minutes, the alcohol separated by filtering through a cloth and the solid material finally pressed to a hard mass by a laboratory hand press. In either case, the dried tissue was passed through a coffee mill and a fine powder obtained.

(2) The dried tissue was thoroughly extracted several times (generally four to six) with excess of absolute alcohol, the various extracts mixed and concentrated to small bulk under reduced pressure at 40°.

(3) The residue was taken up with a small volume of ether, in which much of it remained insoluble. To the mixture, without any attempt at

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filtration, acetone was added in excess and the precipitate obtained pounded together by a pestle and separated. The precipitate was again mixed with ether, precipitated by acetone and treated as before. By this process, which was repeated three times, all but traces of acetone-soluble bodies were removed.

(4) The precipitate from above was rubbed up in a mortar with a large excess of water and a good emulsion made; to the emulsion from one-quarter to one-third of its volume of acetone was added. On the addition of the acetone a large amount of substance separated in the form of large white flakes and floated on the surface of the liquid. This was removed partly by means of a glass spatula, and partly by filtration. It was again emulsified and precipitated three times more. The filtrate obtained from the first emulsification always contained much soluble matter—the nitrogenous impurity previously referred to—but after the first treatment only traces of this impurity were found in the liquid.

The solid substance which separated was now dried by treating it several times with fresh additions of acetone. Finally, as much acetone as possible was pressed out of the mass by means of a pestle and the whole taken up in ether, in which it was still partly insoluble, forming an opalescent mixture.

(5) The ether mixture was centrifuged when a clear supernatant fluid and a white precipitate were obtained. This white precipitate was found to be impure sphingomyelin. The ethereal solution was decanted off and treated with excess of acetone. The resulting precipitate was again taken up with ether when an almost clear solution was obtained. Centrifuging was repeated as before and the clear supernatant ethereal solution again precipitated with acetone. Generally it was only necessary to centrifuge twice, but if the phosphatide was not quite soluble in ether after a second treatment the process was repeated until a precipitate was obtained which was quite soluble in ether.

(6) The substance was now dissolved in alcohol, and if the solution was not quite clear it was allowed to stand for some time until the insoluble substance (crude kephalin) settled on the bottom of the flask. The amount of alcohol-soluble substance obtained depended on circumstances already explained. After separation of the alcohol-insoluble part by decantation the solution was filtered.

(7) The solution was now evaporated under reduced pressure at 40°, the residue taken up with ether, and the phosphatide separated from the

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ethereal solution by acetone. The precipitate obtained gave a perfectly clear solution with ether and with alcohol. It was treated with acetone several times and dried in the desiccator *in vacuo* over sulphuric acid. This substance had all the properties of "lecithin." The samples obtained contained about 1·85 % of nitrogen and 4 % of phosphorus and had a N : P ratio of approximately 1 : 1.

Distribution of nitrogen in "lecithin."

At first, attempts were made to estimate the amount of nitrogen obtained by Van Slyke's method [1912], using the unhydrolysed lecithin. These experiments were rendered difficult, partly on account of the frothing which resulted, and partly owing to the difficulty of finding a suitable solvent for the lecithin. The solvent used was strong acetic acid, but it was found that after the preliminary shaking of the sodium nitrite and acetic acid according to Van Slyke's instructions, the addition of acetic acid (instead of the usual aqueous solvent or very weak acid or alkali) acted in such a way as to give a good deal of gas which was not taken up by the permanganate absorbing mixture. This introduced an error which had to be allowed for by controls, but even then the results were not satisfactory. In spite of these disadvantages, however, the numbers obtained are very suggestive. On hydrolysing the lecithin and then using the liquid containing the soluble products of hydrolysis no difficulty whatever was experienced.

The choline was estimated by hydrolysis with weak acid followed by the steps already described.

Experiments with whole "lecithin."

(1) *Amino nitrogen.* After dissolving the "lecithin" in strong acetic acid the solution was allowed to stand for 24 hours; unless this was done concordant results could not be obtained, for a fresh solution of "lecithin" in acetic acid invariably gave too high results. The estimation was done exactly as described by Van Slyke, secondary octyl alcohol being used to prevent frothing as much as possible.

Experiment. 2·35 g. "lecithin" dissolved in 25 cc. strong acetic acid; different amounts of this were taken and amino nitrogen estimated.

(1) 5 cc. (equal to 0·47 g. "lecithin") = 4·9 cc. nitrogen at 20°, 769.

(2) 5 cc. (equal to 0·47 g. "lecithin") = 4·95 cc. nitrogen at 20°, 769.

(3) 4 cc. (equal to 0·376 g. "lecithin") = 3·9 cc. nitrogen at 20°, 769.

Therefore 1 g. "lecithin" contains 6·0097 mg. N as amino nitrogen = 31·4 %.

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(2) *Choline nitrogen.* 0·9714 g. "lecithin" boiled for several hours with N/10 H_2SO_4, filtered, treated with $Ba(OH)_2$, filtered, residue taken up with alcohol and made up to 25 cc.

10 cc. gave 0·1040 g. choline Pt. chloride = 66 % of total nitrogen.

The nitrogen in this sample was distributed as follows.

N in fatty acids	=	4 %
N as choline	=	66 %
N in amino form	=	31·4 %
Total	=	101·4 %

Other results obtained in this way are shown in the table.

No.	Amino nitrogen found in percentage of total nitrogen	Choline nitrogen found in percentage of total nitrogen	Nitrogen retained by fatty acids in percentage of total nitrogen	Percentage of total nitrogen accounted for
1	30	68·6	3	101·6
2	38·2	63	4·4	105·6
3	28	64	6	98
4	30	68	6	104

Although these results are not altogether satisfactory, it must be remembered that there are great difficulties in getting exact quantitative results when using platinum chloride as a precipitant for choline; also the very small amount of nitrogen retained in the fatty acids is naturally difficult to estimate accurately. When to these are added the difficulties experienced in dealing with whole "lecithin" in Van Slyke's apparatus the results are as accurate as could be reasonably expected. They suggest that all the nitrogen of "lecithin" is present, partly as choline, and partly in the amino form—as amino-ethyl alcohol. This is better brought out in the next set of experiments, where hydrolysed lecithin was used.

Experiments with hydrolysed "lecithin."

In these experiments hydrolysis was carried out with dilute hydrochloric or sulphuric acid. Since the fatty acids always retain a small amount of nitrogen, and the nature of this small fraction of the total is difficult to ascertain, the investigation was limited to the determination and distribution of the filtrate nitrogen. On account of technical considerations it was found most convenient in some cases to estimate the choline in one portion of the lecithin, a separate portion of the same sample being used for the amino-ethyl alcohol determination. This was done in two experiments. In other cases aliquot parts of the same filtrate were taken.

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(1) *Amino nitrogen.* For the determination of this fraction hydrochloric acid was used. About 1 g. of the "lecithin" was boiled with excess of the acid for several hours. After separation of the fatty acids they were thoroughly washed several times with hot water, separated by filtration, and the combined extracts evaporated to a small bulk on the water bath. The hydrochloric acid was then nearly neutralised with sodium hydroxide, the liquid filtered, the residue extracted with water and the total filtrates made up to a definite volume (10–20 cc.). Of this 2 cc. were taken and the amino nitrogen estimated by Van Slyke's micro-apparatus.

(2) *Choline nitrogen.* For the quantitative determination of choline N/10 H_2SO_4 was used, the process being carried out in the manner already described. The results of two experiments are given below.

No.	Source of Lecithin	Percentage of filtrate nitrogen recovered as choline	Percentage of filtrate nitrogen recovered as amino-ethyl alcohol	Percentage of nitrogen accounted for
1	Egg yolk	70	31	101
2	"	66·4	35·8	102·2

Experiments with "lecithin," using aliquot parts of the same filtrate.

1·0935 g. lecithin were boiled with 100 cc. N/5 H_2SO_4 for 48 hours. After separation of the fatty acids the usual manipulations were performed and a final alcoholic filtrate of 25 cc. obtained. Separate portions of this were taken for the determination of (1) choline, (2) amino-ethyl alcohol, (3) total nitrogen.

(1) *Determination of choline.* To 10 cc. of filtrate an alcoholic solution of platinum chloride was added. The precipitate obtained after drying to constant weight at 105° weighed 0·115 g. This figure was obtained after deducting the weight of the double salt of amino-ethyl alcohol platinum chloride which was present to the extent represented by 0·07 mg. amino-nitrogen.

(2) *Determination of amino-nitrogen by Van Slyke's method.* 10 cc. alcoholic filtrate were acidified with hydrochloric acid and carefully evaporated to dryness. The residue was dissolved in 2·5 cc. distilled water, 2 cc. of this being used for the determination.

2 cc. aqueous solution gave 2·55 cc. N at 20° and 755,
i.e. 10 cc. contained 1·802 mg. amino-nitrogen.

(3) *Determination of total nitrogen.* 3·5 cc. alcoholic filtrate required 1·75 cc. N/10 H_2SO_4 = 2·45 mg. N, i.e. 10 cc. = 7 mg. N.

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From these figures:

Total nitrogen in filtrate = 17·5 mg.

Nitrogen present as choline	=	13·07 mg.
Nitrogen present as amino-ethyl alcohol	=	4·505 mg.
Total		17·575 mg.

In this case the nitrogen of the filtrate was all accounted for, 74·6 % being present as choline and 25·7 % as amino-ethyl alcohol.

Somewhat similar results were obtained in other experiments as shown by the table.

No.	Source of Lecithin	Total nitrogen of filtrate in mg.	Choline Pt. chloride obtained in g. (calculated from amount obtained from aliquot part)	Mg. nitrogen present in choline chloride obtained	Mg. nitrogen present as amino-ethyl alcohol	Percentage nitrogen as choline	Percentage nitrogen as amino-ethyl alcohol	Percentage of total filtrate nitrogen accounted for
1	Egg yolk	16·5	0·2860	13	3·9	78·8	23·6	102·4
2	Ox heart	20·2	0·3298	15	4·95	74·2	24·5	98·7
3	Horse kidney	23	0·3364	15·3	8	66·5	34·8	101·3

From these results there can be no doubt that the whole of the water-soluble nitrogen of lecithin is present in the form of choline and amino-ethyl alcohol. It is probable that the nitrogen retained in the fatty acids is also in the amino form though some of this may be due to traces of impurity in the lecithin; it is worth noting that in many cases the nitrogen content of the lecithin is often slightly higher than is required to give a N : P ratio of 1 : 1. At any rate the part retained by the fatty acids is small.

When an alcoholic solution of choline containing amino-ethyl alcohol is precipitated by platinum chloride it frequently happens, if the solutions are concentrated, that some amino-ethyl alcohol platinum chloride is carried down with the choline double salt. This was found to be the case to a very slight extent in the above experiments. Here the amino nitrogen actually present in the platinum chloride precipitate was estimated and due allowance made. In this connexion, it is best not to have the alcoholic choline solution too concentrated; again the precipitate should be very thoroughly washed with absolute alcohol before drying. It must be dried at 105° to remove water of crystallisation. In pure alcoholic solution amino-ethyl alcohol gives a distinct precipitate with platinum chloride, especially if the latter is added in excess, but in the presence of glycerol and other impurities the double salt tends to remain in solution[1].

[1] In this connexion Osborne and Wakeman [1915] record the fact that the final alcoholic extract of some hydrolysed lecithin yielded on the addition of platinum chloride only a certain

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Experiments with phosphatides differing in solubility in alcohol.

When lecithin is prepared from a tissue and kept for some time, a good deal of it tends gradually to become insoluble in alcohol while some remains easily soluble as already described. Between these two extremes, however, fractions of varying solubility in alcohol can be obtained.

Thus, in one experiment about 20 g. of some old heart lecithin were rubbed up three times in a mortar with about 80 cc. alcohol. The alcohol was separated by filtration and contained lecithin readily soluble in alcohol which was separated and dried in the usual way: Fraction I. The solid residue from above was now extracted thoroughly with excess of alcohol, and a lecithin fraction rather less soluble in alcohol obtained: Fraction II.

The alcohol-insoluble part remaining was dissolved in ether and great excess of alcohol added. A great deal was precipitated but some remained in solution: Fraction III.

The precipitate was dissolved in ether and precipitated by acetone. This was practically alcohol-insoluble kephalin: Fraction IV.

From the results already obtained it was to be expected that the amount of choline present should decrease and the amount of amino-ethyl alcohol increase in proportion to the relative insolubility in alcohol of the different fractions.

This proved to be the case; the results given were obtained by the methods already described.

Phosphatide	Percentage of nitrogen as choline	Percentage of nitrogen as amino-ethyl alcohol
Fraction I	67·7	33
„ II	—	38·2
„ III	43	55·3
„ IV	—	81

These figures substantiate the statement previously made that "lecithin" is a variable *mixture* and not a *compound*. Osborne and Wakeman in the communication already referred to state that the ether-soluble milk phosphatide obtained by them may be a mixture or combination of two substances, one yielding choline and the other a base containing amino-nitrogen. They intend to fractionate the substance to settle this question. In the light of the above observations it is probable that this phosphatide will be found

fraction of the total choline; after evaporating the filtrate, taking the residue up in alcohol and adding more platinum chloride another precipitate was obtained. This was repeated four times. It is probable, however, that some of these latter precipitates at any rate contained amino-ethyl alcohol platinum chloride.

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to fall into line with other phosphatides in that it consists of a mixture of true lecithin and kephalin.

Attempts were now made to separate "lecithin" into its component parts. After many unsuccessful experiments I succeeded in doing this, and obtained a true lecithin with all its nitrogen present as choline.

Separation of "lecithin" into its components.

The lecithin used was derived from ox hearts and from eggs, the extraction and purification being carried through as quickly as possible.

The first experiments described were conducted with ox heart lecithin which was at first completely soluble in alcohol. On analysis for nitrogen, phosphorus and distribution of nitrogen, it gave the following results.

Nitrogen (Kjeldahl). 0·8007 g. required 10·85 cc. N/10 H_2SO_4 = 1·89 % N.

Phosphorus (Neumann). 0·5990 g. required 43·7 cc. N/2 NaOH = 4·04 % P.

N : P = 1·03 : 1.

Distribution of nitrogen.

1·3202 g. lecithin boiled with 120 cc. N/10 H_2SO_4 for 48 hours gave, after the usual treatment and addition of platinum chloride to the final alcoholic solution, 0·3673 g. choline platinum chloride (platinum content = 31·63 %).

The fatty acids contained 0·448 mg. nitrogen.

From above figures:

Total nitrogen of 1·3202 g. "lecithin"	=	24·95 mg.
Nitrogen found in fatty acids	=	0·448 mg.
Nitrogen of filtrate (by difference)	=	24·502 mg.

Now 24·502 mg. nitrogen = 0·5387 mg. choline platinum chloride and choline platinum chloride obtained = 0·3673 mg., i.e. 68 % of filtrate nitrogen is derived from choline.

By Van Slyke's method the filtrate from choline platinum chloride was found to contain 28 % of its nitrogen in amino form.

After standing for four days in an evacuated desiccator this "lecithin" formed a slightly opalescent alcoholic solution which on standing for some time deposited a very small amount of a sticky brown substance. The

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solution was filtered and treated with excess of an alcoholic solution of cadmium chloride.

The resulting precipitate of cadmium chloride compound was thoroughly washed with alcohol, the latter being finally drained off by means of the suction pump. The precipitate was then dried *in vacuo*.

The dried powder was now ground up in a mortar with excess of pure dry ether. By this means it was hoped to extract the kephalin fraction, since kephalin cadmium chloride is said to be soluble in ether, while, on the other hand, the corresponding lecithin salt is supposed to be insoluble.

Here, however, the whole of the cadmium chloride precipitate dissolved in ether to form an almost clear solution. The ether was partly evaporated under reduced pressure; the concentrated solution obtained being spread out in a thin layer under a fan and freed from ether. The residue was taken up with fresh ordinary ether which had not been specially purified; a good deal remained in suspension as a brownish white powder. This was separated by the centrifuge and dried; it was then thoroughly ground in a mortar, extracted with ether several times and finally obtained as a white powder.

The ether-soluble part was freed from ether and the residue finely powdered. On treating a fraction of this with ether it was found to be entirely ether-soluble.

The cadmium chloride compound was now divided into two parts:

A. An ether-insoluble part forming a white powder.

B. An ether-soluble part forming a brownish white powder.

Treatment of ether-insoluble fraction A.

This fraction was further purified by twice recrystallising it from a mixture consisting of ethyl acetate, 2 parts, 80 % alcohol, 1 part.

The compound was dissolved by heat in this mixture and on cooling the greater part separated as a white precipitate, which under the microscope showed beautiful white clusters of well-formed needles arranged in stars. These crystals were washed with ethyl acetate and with ether and dried.

Analysis of crystals.

Nitrogen (Kjeldahl). 0·2998 g. required 3·1 cc. N/10 H_2SO_4 = 1·45 % N.

Phosphorus (Neumann). 0·1310 g. required 7·45 cc. N/2 NaOH = 3·15 % P.

N : P = 1·02 : 1.

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Distribution of nitrogen in crystals.

0·5896 g. was boiled with excess of N/10 H_2SO_4 for 42 hours, the final alcoholic solution containing the soluble nitrogen being obtained in the usual way. Filtrate gave 0·1644 g. platinum chloride salt (Pt. content 31·55 %). Fatty acids contained 0·95 mg. nitrogen.

From above figures:

Total nitrogen of 0·5896 g. compound	= 8·54 mg.
Nitrogen found in fatty acids	= 0·95 mg.
Filtrate nitrogen (by difference)	= 7·59 mg.

Now 7·59 mg. nitrogen = 0·1669 g. choline platinum chloride and choline platinum chloride obtained = 0·1644 g. i.e. 98·5 % of filtrate N is derived from choline.

The filtrate from the platinum chloride precipitate gave no amino nitrogen by Van Slyke's method. The whole of the filtrate nitrogen was therefore removed by platinum chloride.

With the exception of the variable amount of nitrogen always retained by the fatty acids this powder contained all its nitrogen as choline, while the "lecithin" from which it was derived contained only 68 % of its soluble nitrogen as choline.

This separation proves that "lecithin" is a mixture from which its choline-lecithin component can be separated by making use of the insolubility of its cadmium chloride salt in ether.

Investigation of the ether-soluble part of the cadmium chloride precipitate (Fraction *B* above) showed that it still contained some choline. Since the whole of the cadmium chloride precipitate originally obtained from lecithin dissolved in ether on the first attempt at separation, this result was to be expected. Choline to the extent of about 30 % of the total nitrogen was found. Attempts to reduce the amount of choline were frustrated by the extreme solubility of the salt in ether, and it is probable that although true lecithin cadmium chloride is insoluble in ether, it dissolves to a certain extent in an ethereal solution of kephalin cadmium chloride.

A fresh sample of lecithin was now obtained from hearts, and the cadmium chloride compound prepared as before. Here the fractionation of the salt gave rise to great difficulties, for when treated with ether an opalescent solution was formed which it was impossible to filter or centrifuge. Evaporating the ether and again taking up the dried substance with ether

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—a procedure which was very effective in the former separation described—proved quite useless here. It was found, however, that if the dried material was made into a thick paste with alcohol, and ether containing 2 % alcohol was used, an opalescent solution was obtained which filtered very well. By this means the compound was divided into two parts as before.

A. Fraction insoluble in ether = true lecithin.

B. Fraction soluble in ether = kephalin contaminated with lecithin.

Now Thudichum [1884] makes the statement that lecithin cadmium chloride is soluble in cold benzene, and that the part which remains insoluble in the cold but dissolves on heating, to fall out again on cooling, is not lecithin but a substance which he calls paramyelin. An attempt at fractionating the white powder *A* by means of benzene was therefore made. This, however, was unsatisfactory and in my case the process did not appear to be of much value. When cold benzene was added to the powder very little indeed appeared to dissolve, but on heating the mixture nearly all the solid disappeared. On leaving this solution to stand in the ice chest for some hours an opalescent layer formed at the bottom with a clear liquid above. On filtration an absolutely clear benzene solution was obtained, while a fair amount of a semitransparent gelatinous substance was left on the filter. When this was treated with hot benzene part of it dissolved but did not form a clear solution. On again leaving it to stand at low temperature a marked deposit formed. This deposit constituted fully one half of the total substance.

When treated with hot benzene some of this apparently goes into solution, and the process of separation of lecithin cadmium chloride into benzene-soluble and benzene-insoluble fractions appears to be unsatisfactory. It seems that practically the whole can be got into solution if sufficiently large volumes of benzene are used and any differences of solubility that may exist are probably due to slight oxidation or other changes; the "insoluble" and "soluble" fractions are probably the same substance.

The ether-insoluble fraction was now divided into

(1) Fraction remaining soluble in cold benzene (original solution being accomplished by means of heat),

(2) Fraction insoluble in cold benzene (soluble more or less in hot benzene).

The cold benzene solution was treated with twice its volume of alcohol and the precipitate obtained separated by the centrifuge.

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The cold benzene-insoluble fraction (2) was spread out in a thin film under a fan and dried at room temperature. Both substances were then dissolved in the ethyl acetate-alcohol mixture as before and white crystalline precipitates obtained. The following scheme represents the fractionation of the original "lecithin."

Lecithin ⟶ $CdCl_2$ compound
- Ether-insoluble fraction
  - Benzene-soluble fraction (lecithin)
  - Benzene-insoluble fraction (lecithin)
- Ether-soluble fraction (impure kephalin)

The distribution of the nitrogen in these compounds is given in the table. Hydrolysis[1] was carried out in the usual way and aliquot parts of the filtrate used for choline and for total nitrogen. The amino nitrogen was estimated in the filtrate after the separation of choline as the platinum salt. No account is taken of the nitrogen retained by the fatty acids or other precipitate.

The numbers refer to the total nitrogen of the filtrate.

No.	Substance	Percentage nitrogen as choline	Percentage nitrogen in amino form	Percentage of total filtrate nitrogen accounted for
1.	Lecithin	62	37·2	99·2
2.	Cadmium chloride lecithin, before ether	65	33	98
3.	Ether-insoluble fraction (benzene-soluble)	94	3	97
4.	Ether-insoluble fraction (benzene-insoluble) ...	99	None	99
5.	Ether-soluble fraction	45	54	99

Here the two fractions (3 and 4) contained all their nitrogen (with the exception of a trace in No. 3) as choline. A cadmium chloride compound of a true choline-lecithin has therefore been obtained from an original lecithin which contained only 62 % of its nitrogen as choline.

It is impossible to get the kephalin part quite free from true lecithin by this process, but kephalin can be obtained in the pure state by treatment with alcohol and suitable purification, and as such has been isolated and

[1] Considerable difficulty was sometimes experienced in hydrolysing cadmium chloride compounds of phosphatides with N/10 H_2SO_4. Often a great deal of the nitrogen was retained in the precipitates obtained during the preparation of the final alcoholic extract for precipitation by platinum chloride. Sometimes the whole of the nitrogen (with the exception of the small amount retained in the fatty acids) was found in the filtrate. The reason of these anomalous results I have been unable to ascertain. When lecithin itself was used no difficulty of this kind was encountered.

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analysed by many investigators. It contains the whole of its nitrogen as amino-ethyl alcohol.

So far, only the cadmium chloride salt of true lecithin had been isolated, but on separation of the free phosphatide by means of ammonium carbonate, free lecithin was obtained which had all its nitrogen in the form of choline. This particular sample of lecithin was derived from egg yolk and as its method of preparation differed considerably in certain respects from the methods already given, this will now be described.

Preparation of a sample of lecithin containing all its nitrogen as choline.

Dried egg yolk was powdered and extracted with alcohol: to the alcoholic extract was added excess of alcoholic solution of cadmium chloride.

The resulting precipitate was washed with alcohol and then rubbed up with about fifteen times its volume of ether containing a trace of alcohol. A dense opalescent mixture was obtained which on centrifuging separated into a brownish deposit and clear supernatant liquid. The deposit was thoroughly washed with ether, dried and decomposed by boiling in alcohol with ammonium carbonate according to the method recommended by Bergell [1900].

The alcoholic solution was concentrated, the residue taken up with ether and excess of acetone added. The precipitate obtained was emulsified with water and treated by the usual process for the purification of lecithin [MacLean, 1912]. The lecithin was dissolved in alcohol and again precipitated by cadmium chloride. The double compound obtained was recrystallised from the ethyl acetate mixture already described. Beautiful white feathery crystals of pure lecithin cadmium chloride were thus obtained.

Analysis of pure lecithin cadmium chloride (egg).

Nitrogen (Kjeldahl). 0·5604 g. required 5·85 cc. N/10 H_2SO_4 = 1·46 % N.

Phosphorus (Neumann). 0·2600 g. required 15·8 cc. N/2 NaOH = 3·37 % P.

N : P = 1 : 1·04.

Test for amino nitrogen.

1·8615 g. were boiled for 10 hours with dilute hydrochloric acid (96 cc. H_2O + 4 cc. conctd. HCl); after cooling the fatty acids were separated by filtration, the filtrate evaporated to a small bulk on the water bath and nearly

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neutralised with alkali. The whole was then made up to 20 cc. Of this 2 cc. were used for each experiment.

The following results were obtained by Van Slyke's method.

Controls with reagents $\left\{\begin{array}{ll}(1) & 0{\cdot}12 \text{ cc. nitrogen} \\ (2) & 0{\cdot}13 \text{ cc. } \quad ,,\end{array}\right\}$ at 752 and 20°.

Experiments $\left\{\begin{array}{ll}(1) & 0{\cdot}14 \text{ cc. nitrogen} \\ (2) & 0{\cdot}15 \text{ cc. } \quad ,, \\ (3) & 0{\cdot}15 \text{ cc. } \quad ,,\end{array}\right\}$ at 752 and 20°.

Average 0·146 cc. nitrogen.

2 cc. therefore contained 0·021 cc. nitrogen and from this 1·8615 g. (20 cc. solution) gives 0·210 cc. nitrogen = 0·117 mg. nitrogen in amino form.

Total nitrogen in 1·8615 g. compound = 27·17 mg.
Total nitrogen in filtrate = 26·80 mg.
Retained by fatty acids, etc. = 0·37 mg.

From above figures:

Percentage of total nitrogen in amino form = 0·430
Percentage of filtrate nitrogen in amino form = 0·437

Separation of pure lecithin from its cadmium chloride compound.

This was done as already described by ammonium carbonate. The substance obtained was thoroughly purified by means of acetone and water and finally dried *in vacuo* over H_2SO_4.

Analysis.

Nitrogen (Kjeldahl). 0·3747 g. required 5 cc. N/10 H_2SO_4 = 1·87 % N.
Phosphorus (Neumann). 0·2710 g. required 20·3 cc. N/2 NaOH = 4·15 % P.

N : P = 1 : 1.

Estimation of choline.

1·8426 g. were boiled with 150 cc. N/10 H_2SO_4 and after the necessary manipulations 25 cc. of a final alcoholic solution obtained.

10 cc. of this solution gave 0·2993 g. platinum salt (platinum content 31·7), therefore total filtrate = 0·7483 g. platinum salt.

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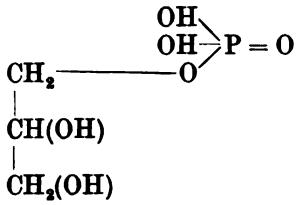
Now total nitrogen of 1·8426 g. substance = 34·46 mg. = 0·7581 g. choline platinum chloride; obtained 0·7483 g. choline platinum chloride, i.e. 98·7 % of total nitrogen is derived from choline.

Since, however, a small amount of nitrogen is always retained in the fatty acids the whole of the soluble nitrogen of this specimen may be regarded as choline. The platinum chloride removed every trace of the filtrate nitrogen.

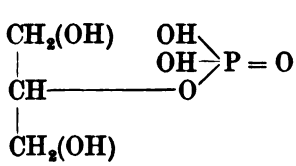
Some considerations on the chemical constitution of "lecithin."

Fatty acids. The fact that lecithin has been shown to consist of two components throws some light on the difficulties experienced by many investigators when endeavouring to ascertain the nature of the fatty acids present. Theoretically only two acid radicles exist in the molecule, and separation and identification ought to be easy, but, generally, mixtures are obtained which appear to consist of more than two acids. It is hoped, however, that examination of the true lecithin obtained by the processes described will give better results.

Glycerophosphoric acid. From the chemical standpoint this acid can exist in two modifications—the α- and β-forms.



α-form (unsymmetrical)



β-form (symmetrical)

The α-form contains an asymmetric carbon atom and is therefore optically active, while the β-variety is inactive.

After the discovery by Willstätter and Lüdecke [1904] that the glycerophosphoric acid of egg lecithin was optically active, rotating the plane of polarisation of polarised light to the left, it was generally assumed that the acid present in lecithin was α-glycerophosphoric acid, though Tutin and Hann [1906] disputed this and held that lecithin glycerophosphoric acid consisted of a mixture of the α- and β-forms. Recently Grimbert and Bailly [1915] claim to have definitely shown that egg lecithin is a mixture of at least two isomers containing the symmetrical and unsymmetrical varieties of glycerophosphoric acid.

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It is possible that the true lecithin part of "lecithin," containing all its nitrogen as choline, may have one form of this acid, while the kephalin part may have the other form. Some experiments bearing on this point were begun some time ago but have had to be stopped for the present. I hope, however, to take up this point in the future and to settle some of these problems in a later communication.

Summary.

(1) When phosphatides are extracted from tissues by means of alcohol they invariably contain large amounts of a nitrogenous impurity. This is very difficult to remove by any of the ordinary methods for preparing "lecithin" and many bodies described as new substances are simply "lecithin" contaminated with this body.

(2) This nitrogenous impurity is very complex chemically and contains bodies of a purine nature.

(3) The whole of the nitrogen of "lecithin" is accounted for by the choline and amino nitrogen present.

(4) "Lecithin" is a mixture in variable proportions of two somewhat similar substances, one having the whole of its nitrogen in the form of choline —true lecithin—while the other contains no choline but has all its nitrogen represented by amino-ethyl alcohol—kephalin.

(5) Reasons for the divergent statements as to the occurrence of kephalin in different tissues are given.

(6) By fractionation of the cadmium chloride salt of "lecithin" with ether, "lecithin" has been divided into its two components—true lecithin and kephalin. True lecithin in which every trace of the nitrogen was present as choline was obtained. The kephalin fraction contained a certain amount of choline, but had the greater part of its nitrogen as amino-ethyl alcohol.

(7) Various points on the mode of occurrence, distribution and purification of the phosphatides are discussed.

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XXXVII. THE REDUCING ENZYME OF *BACILLUS COLI COMMUNIS.*

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A reducing enzyme is fairly widespread in the tissues of animals and plants and has of late received the attention of several workers in the province of enzymic chemistry. The results obtained independently by some of the investigators seem to show that the so-called reductase is not a single principle but a system. Thus Bach [1911] working on the reducing enzyme in the calf's liver was able by treating the tissue with a 2 % solution of sodium fluoride and pressing it through a cloth to obtain an inactive filtrate and a residue which when emulsified with water either did not reduce methylene blue at all or did it extremely slowly. On the addition of 1 % of acetaldehyde to the emulsified residue the reducing activity was restored. Harden and Norris too [1914] showed that dried yeast and zymin lost their power of reducing methylene blue and sodium selenite when washed. This loss of the reducing power they found could be restored by the addition of the washings, bouillon and some aldehydes. Recently [1915] they have observed a similar phenomenon with the reducing enzyme of muscle. Schardinger's observation that the addition of acetaldehyde and formaldehyde to milk imparts to it the power of reducing methylene blue to its leuco-base is another illustration of the complexity of reductase.

The authors find that *B. coli communis* (Escherich) when washed will not reduce methylene blue, but that it acquires the power of reduction on the addition to it of various reagents incapable by themselves of effecting the reduction. This property of the organism in question was investigated and the results obtained form the subject of this communication.

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The following technique was adopted in the preparation of the bacterial emulsion used in the experiments. Roux bottles containing nutrient agar were inoculated with a 24 hours' broth culture of *B. coli*. The organism was then grown for 48 hours at 37° after which period it was washed off the agar with saline, a glass scraper being used in order to ensure a more thorough removal of the growth. The resulting emulsion was then centrifuged and after removing the supernatant saline fresh salt solution was added, the organisms stirred up, and again centrifuged. This process of washing was repeated two or three times and the residue was finally diluted with saline (the growth from six Roux bottles to 50 cc. of saline) and shaken up with glass beads in order to obtain an even suspension of the bacilli. The emulsion was then ready for use. With very few exceptions a fresh preparation was made daily. If kept in the cold room, however, the emulsion retained its reducing power for some days.

As already mentioned *B. coli* will not reduce methylene blue when washed in saline unless certain substances are added to it. A great number of reagents were tested for this property and a good many of these yielded positive results. The following list comprises the substances tried.

Positive.	*Negative.*
Washings	Acetaldehyde
Bouillon	Formalin
Sodium lactate	Lactose
Sodium pyruvate	Sorbitol
Sodium glycerate	Dulcitol
Glucose	Raffinose
Galactose	Erythrol
Laevulose	Rochelle salt
Mannose	Glycocoll
Maltose	Potassium oxalate
Arabinose	Anisaldehyde
Mannitol	Oenanthol
Sodium succinate	Phloroglucinol
Sodium formate	Propaldehyde
Alanine	Isovaleraldehyde
Asparagine	Cinnamyl aldehyde
Xylose	Salicylaldehyde
Inulin	Hydroxybutyric acid
Sodium acetate	Butyl alcohol
Isobutyric acid	Linolenic acid
Globulin	Sodium citrate
Albumin	Hydantoin
Horse serum	Creatine
Peptone (Witte)	Guanine hydrochloride
Liebig's Extract	Pyridine
	Hypoxanthine } In suspension
	Adenine } In suspension
	Xanthine } In suspension

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In carrying out the above experiments the following quantities were used: 1 cc. of the bacterial emulsion, 1 cc. of the reagent, 0·2 cc. methylene blue (5 cc. saturated solution diluted to 200 cc. with water). The mixtures were made up to 5 cc. with saline. The salts were employed in N/10 solution, while the other substances, with the exception of the washings, broth, and horse serum, were in 1 % solutions or suspension. All the reductions were carried out at 35–37°. The time taken to reduce the methylene blue varied with the reagent used. The results were regarded as negative when no reduction was observed within an hour. The addition of broth produced one of the highest rates of reduction; an addition of a very small quantity sufficed to impart the reducing power to the emulsion. This is shown by the following experiment:

Emulsion cc.	Broth cc.	Saline cc.	Methylene blue cc.	Time taken for reduction mins.
1	—	4	0·2	(no reduction)
1	0·1	3·9	0·2	20
1	0·2	3·8	0·2	14·5
1	0·3	3·7	0·2	12
1	0·4	3·6	0·2	11·5

It has been suggested that in the process of reduction water is split into H and O and in order that the reduction should occur, the presence of an acceptor for the oxygen is necessary. The great variety in the chemical character of the reagents in the list which gave positive results does not afford definite information as to the precise chemical properties requisite for an acceptor. Bach [1912] in connection with his work on the reductase in calf's liver, discussing the chemical nature of the acceptor or coferment, as he calls it, suggested the possible presence in all solutions capable of acting as acceptors of aldehydes formed from such degradation products of the metabolism of the proteins as α-amino acids. In support of his suggestion he refers to Strecker's observations that α-amino acids are oxidised at ordinary temperature by alloxan forming aldehydes of a series containing fewer carbon atoms and giving off at the same time CO_2 and NH_3, a process which might possibly occur in nature. The experiments quoted above however show that in the case of *B. coli* all the aldehydes tried with the exception of some of the sugars are incapable of acting as acceptors under the conditions employed. From the same experiments we can also conclude that the reducing enzyme in the organism in question differs to some extent from those present in the liver of the calf and in yeast, since in the former case according to Bach acetaldehyde can act as acceptor and

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in the latter case according to Harden and Norris salicylaldehyde, benzaldehyde, anisaldehyde, and isovaleraldehyde are able to restore the reducing power to washed yeast.

The effect of the addition of increasing quantities of broth was next studied and it was found that the rate of reduction increased at first with the increase in the quantity of broth added until a certain optimum was reached, after which any further increase in the quantity of broth added decreased the rate of reduction. This is illustrated by the following experiment.

Emulsion cc.	Broth cc.	Saline cc.	Methylene blue cc.	Time of reduction mins.
1	—	4	0·2	(not reduced)
1	0·1	3·9	0·2	28·5
1	0·2	3·8	0·2	24
1	0·3	3·7	0·2	22·5
1	0·4	3·6	0·2	19
1	0·5	3·5	0·2	14·5
1	1	3	0·2	15
1	1·5	2·5	0·2	15·5
1	2	2	0·2	18
1	2·5	1·5	0·2	20
1	3	1	0·2	20·5
1	3·5	0·5	0·2	24

This decrease in the rate of reduction produced by increased addition of broth after a certain optimum is an observation of some interest. Palladin [1914] found that the addition of glycerol and pyridine to hefanol produced a deterrent effect on the activity of the reductase in it and that the extent of the retardation varied with the amount of the reagent added. He ascribes the fact to the displacement of the water by the glycerol and pyridine. Pyridine does not act as an acceptor when added to *B. coli*, glycerol on the other hand does, but its behaviour was observed to be similar to that of broth, since it has at first an activating influence which is proportional to the amount added, but after a certain point any increase in the amount of the reagent decreases the rate of reduction. This is shown in the following experiment.

Emulsion cc.	Glycerol 50 % cc.	Saline cc.	Methylene blue cc.	Time of reduction mins.
1	—	4	0·2	(no reduction)
1	1	3	0·2	20
1	2	2	0·2	16·75
1	3	1	0·2	20·5
1	4	—	0·2	23

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This experiment and also the one carried out with the broth suggest that both reagents possess an activating, as well as a retarding action on the reductase of *B. coli*. The experiments throw no direct light on the cause of the retarding influence, but at the same time they do not exclude Palladin's explanation. It is quite conceivable in this case that glycerol for instance can act chemically as an acceptor, while as it displaces the water owing to its physical condition it can also retard and even stop the action of the reducing system of which it forms a part (i.e. acceptor). It is also plain from the experimental data that initially the rate of reduction is influenced more by the activating than by the retarding factor, but as the quantities of the reagents added are increased the reverse takes place, and this accounts for the decrease in the rate after a certain amount of the reagent has been added.

It was of further interest to investigate how the time of reduction is influenced by varying the amounts of methylene blue and emulsion respectively, and accordingly experiments were instituted with that object. In the first set the amount of emulsion was kept constant and the quantity of methylene blue changed. In the second set the methylene blue was kept constant and the amount of emulsion changed. The following are the results obtained:

	Emulsion cc.	Broth cc.	Saline cc.	Methylene blue cc.	Time of reduction mins.
1.	1	1	2·9	0·1	19
	1	1	2·8	0·2	21·5
	1	1	2·7	0·3	25
	1	1	2·6	0·4	26·5
	1	1	2·5	0·5	29
2.	0·1	1	3·9	0·2	43
	0·3	1	3·7	0·2	20·5
	0·5	1	3·5	0·2	13
	1	1	3	0·2	7·5
	2	1	2	0·2	5

As it is seen from the figures the time taken to reduce increases as the quantity of methylene blue is increased, while in the case of the varying quantities of emulsion the time decreases with the increase in the amount of emulsion.

No essential difference in the results could be observed when some of the experiments were carried out anaerobically, i.e. by boiling the saline previous to using it and by displacing the atmosphere in the tubes and the dissolved oxygen in the emulsion by nitrogen.

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Summary.

1. *B. coli* does not reduce methylene blue when washed and suspended in saline, but does so on addition of certain reagents.

2. A wide variation in chemical nature is exhibited by the reagents capable of acting in this way.

3. The effect of the addition of various amounts of broth and glycerol respectively show that there are two factors concerned in the effect of those reagents which influence the process of reduction, i.e. an activating factor and a retarding one. The former is thought to be chemical while the second is possibly physical.

4. The time of reduction of a certain quantity of methylene blue decreases with increase in the amount of emulsion, while as the amount of methylene blue is increased for a constant amount of emulsion, the time of reduction increases.

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XXXVIII. ON THE DIAZO REACTIONS OF HISTIDINE AND TYROSINE.

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(*Received August 19th, 1915.*)

The test for histidine based on its property of forming a diazo compound, first described by H. Pauly [1904], is one of the most important reactions in the investigation of protein decomposition products. This diazo reaction can be carried out without any modification whether histidine is in the free state or bound up in a protein molecule. Its importance is, however, lessened considerably as it is not quite specific, since tyrosine, too, is capable of coupling with diazobenzenesulphonic acid in alkaline solution and yields a colouration so very similar to that given by histidine, that the differentiation of the two amino acids from each other by means of this reaction is a matter of extreme difficulty. On addition of diazobenzenesulphonic acid in sodium carbonate solution, histidine gives a dark cherry red colouration which, even on dilution with much water, retains its red tone and does not change into yellow: in acid solution it gives a pure orange colouration. As regards tyrosine, the colour in alkaline solution is like that of histidine, but of a less deep red, and, moreover, on dilution, a yellow colour appears, yielding finally an impure yellowish red colour.

Though Pauly was able to describe the difference of the diazo colours produced by histidine and tyrosine, as above, he thought it necessary to mention further, that it is sometimes not easy to distinguish the colours from each other.

Applying the diazo reaction as a test for methyltyrosine, E. Friedmann and S. Gutmann [1910] tried to distinguish the colouration given by this substance from that of histidine by taking into consideration not only the

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colours of the solutions themselves, but also the colours with which these solutions dye filter paper. The histidine compound gives either a pure orange or a lemon yellow colour on paper, depending on the reaction of the solution being alkaline or acid respectively. The methyltyrosine compound stains paper red brown in alkaline, yellow brown in acid solution. Even using this method of differentiation for tyrosine, it is still scarcely possible to distinguish it from histidine by the diazo reaction.

Since, according to Pauly, among the protein cleavage products, histidine and tyrosine alone give the diazo reaction, it is quite evident that the reaction will be more useful in its application, if it be made specific for only one of these two amino acids.

Lately K. Inouye [1912] has succeeded with the following useful modification. He noticed that when tyrosine and histidine are submitted to the Schotten-Baumann reaction, the former is converted into its dibenzoyl derivative and loses its property of coupling with diazotised sulphanilic acid, while the latter, though forming its monobenzoyl compound, still maintains unaltered its ability of yielding a red colouration in the presence of the diazonium acid. He therefore treated the solution to be tested by means of the diazo reaction with benzoyl chloride and sodium carbonate, in order to prevent the tyrosine from combining with the diazo acid, and thus made the diazo reaction specific for histidine.

According to a recent paper by A. Kossel and S. Edlbacher [1915], when histidine loses its free carboxyl group, as, for instance, in its methyl ester, the imidazole ring to which the diazo reaction owes its appearance is readily opened on benzoylation with formation of a tribenzoyl-triamino-acid.

As regards histidine in a protein molecule, where it is to be found in a peptide linking united through its carboxyl group with other amino acids, it is most probable that by treatment with Schotten-Baumann's reagent an analogous transformation of histidine will take place, just as in the case of its methyl ester, resulting in the disappearance of the property of giving the diazo reaction.

In order to apply Inouye's modification of the histidine test to protein it is therefore necessary, before performing the test, to hydrolyse the protein by means of acid or trypsin, so as to obtain the histidine in the free state.

Recently, in applying some new methods of investigation to protein decomposition products, I met with some difficulty in distinguishing these two amino acids by means of the diazo reaction, since, owing to the special circumstances, neither Inouye's test for histidine, nor Millon's reaction could

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be successfully used. At any rate it seemed to me of interest to discover a method of differentiating the diazo reaction as applied to tyrosine and histidine without recourse to benzoylation.

I find a ready distinction may be made by reducing the coloured solutions obtained by Pauly's reaction from these two amino acids. Although the two solutions obtained with diazobenzenesulphonic acid in sodium carbonate solution are at first almost identical in colour, yet on acidifying with hydrochloric acid and reducing with zinc dust, and then adding excess of ammonia, the resulting fluids are quite different in appearance.

The test is applied as follows: a few cubic centimetres of the solution to be tested are placed in a beaker and made alkaline with excess of sodium carbonate solution. A few centigrams of the freshly prepared diazobenzenesulphonic acid are dissolved in a few cubic centimetres of sodium carbonate solution. This solution must always be freshly prepared just before performing the test, as if it be allowed to stand longer than a quarter of an hour, the result of the reaction is sometimes dubious. The use of too large an excess of the reagent should be avoided, otherwise the solution remains slightly yellowish in colour after reduction and the test is occasionally rendered less distinct.

The reagent is now added to the amino acid solution in the beaker. Immediately, or not later than three minutes afterwards, the fluid assumes a dark red colouration if histidine or tyrosine be present. I will refer to this well-known diazo colouration as the primary colouration for the sake of distinguishing it from the colouration produced after reduction. As I have mentioned above, it is extremely difficult to judge by means of this primary colouration, whether the result is due to histidine or tyrosine.

To the coloured solution concentrated hydrochloric acid is added to make it distinctly acid, and this, in each case, changes the colour to a rather yellow shade of orange. The acid solution is reduced by zinc dust for a quarter of an hour or longer, a colourless solution always resulting. A few cubic centimetres of the reduced solution are now transferred to a test-tube by decantation, or, if necessary, by filtering, and then made strongly alkaline by addition of ammonia of about 25 % strength to the extent of approximately twice the bulk of the solution. Of course, the amount of ammonia necessary depends on the quantity of hydrochloric acid and of zinc used, but the above quantities will generally be found sufficient.

If the primary diazo reaction be due to histidine, the fluid yields immediately, or within a few minutes, a very characteristic golden yellow

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colouration of great intensity which is permanent for a long time and is unaltered even on boiling. By the action of hydrogen peroxide on this golden yellow solution, the shade is turned into a beautiful lemon yellow.

The colouration produced by tyrosine in the above reaction is yellowish brown with a small quantity of ammonia, and on gradual addition of ammonia to the amount mentioned above is readily changed to a beautiful bright rose red colouration. This colour, too, is unaltered on boiling, but when left for a while becomes markedly yellow in tint and gradually changes to a reddish brown, which, however, may be reconverted into the former colour by further addition of ammonia or by shaking. Unlike that of histidine, the rose red colouration of tyrosine is remarkably unstable to the action of hydrogen peroxide, so that after oxidation by this substance, the solution will be colourless or, at most, of a faint impure rust colour.

The colouration obtained by the addition of ammonia after reduction of the primary coloured compounds will be called the secondary colouration. The intensity of the colour in the secondary reaction is always proportional to that of the primary colouration.

Converting thus the closely resembling primary colourations of the histidine and tyrosine diazonium compounds into the secondary, which differ so markedly in appearance—the one being golden yellow and the other rose red—one can now very readily distinguish the two amino acids by the diazo reaction followed by the reaction giving rise to the secondary colourations.

As regards histidine, the primary diazo reaction can be applied to a solution as dilute as one part of the substance to 100,000 parts of water; the secondary colouration, too, is obtained as a pale yellow even at this dilution. The peculiarly characteristic deep yellow, however, can be obtained only with a solution stronger than 1 : 20,000.

Glycerol, alanine, cystine, taurine, α-aminobutyric acid, valine, arginine, leucine, lysine, phenylalanine, tyrosine, tryptophane, histidine, proline, aspartic acid, glutamic acid and hippuric acid have been submitted to the test. Serine and oxyproline were not at my disposal.

Not one of these amino acids except histidine was able to produce a golden yellow colouration in the secondary reaction. It appears, therefore, that, at present, this secondary golden yellow colouration may be considered as being given by histidine alone among the protein decomposition products.

The secondary diazo reaction of tyrosine with production of a rose red colouration is so delicate that it can be carried out in a dilution of 1 : 10,000. With a dilution of 1 : 50,000 a faint rose red colouration is obtained. The

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rose red colour, however, is not specific for tyrosine. Cystine, which was carefully purified and freed from tyrosine either by precipitating with phosphotungstic acid or by the method of R. H. A. Plimmer [1913] (treatment with alcohol and hydrochloric acid gas), gives the same colouration as tyrosine, though it is much less intense. Alanine and phenylalanine also slowly produce a faint pink colouration, but the solution must be rather strong. The same colour reaction is also given by asparagine with a delicacy of 1 : 1000. All these colourations are destroyed by oxidation with hydrogen peroxide.

Some organic compounds other than protein cleavage products, for instance, hydroxy-acids such as lactic acid and malic acid, alcohols such as ethyl alcohol, glycol and glycerol, and also acetone, ether and oxamide, behave in a similar manner and yield the pink colouration, with or without the formation of a definite primary colour.

The rose red colouration due to tyrosine in the secondary reaction, therefore, is not specific for this amino acid, but taking into consideration the primary colouration in the diazo reaction which, in the intensity and rapidity of colour formation, is peculiar to tyrosine and histidine, one can be certain that the substance to be tested is really tyrosine when both the first and the second diazo colourations are produced in the particular manner described above. The diazo reaction of tyrosine has sometimes an advantage over Millon's reaction, for the former can be applied with ease, even where the latter is impossible, as for example in the presence of much chloride.

Making use now of the secondary diazo reaction it can be demonstrated clearly that tyrosine and histidine have different affinities for diazobenzenesulphonic acid.

Equimolecular quantities of these two amino acids were mixed together. To one portion of the solution an equimolecular proportion and to another twice this amount of pure diazobenzenesulphonic acid were added in sodium carbonate solution. When the secondary reaction was carried out, the former yielded a golden yellow, and the latter a yellowish brown colouration.

N/100 Histidine in cc.	2	2	2
N/100 Tyrosine in cc.	2	2	2
N/100 Diazobenzenesulphonic acid in cc.	2	4	6
Primary colouration	Brownish yellow	Brown yellow	Yellowish brown
Secondary colouration	Golden yellow	Yellowish brown	Yellowish brown
Colouration after oxidation with hydrogen peroxide	Lemon yellow	Lemon yellow	Lemon yellow

The different colourations produced in this experiment show distinctly that histidine has a greater affinity for diazobenzenesulphonic acid than

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has tyrosine, so that it is the first to couple with the diazo acid. If more than sufficient diazo acid be present to combine with the histidine, the tyrosine enters into reaction and this results in the production of a colouration which is a mixture of the shades produced by the two pure amino acids.

According to the assumption of P. Clemens [1899] that the substance capable of forming the most intensely coloured diazo compound has the greatest affinity for the diazo acid, histidine and tyrosine should possess a greater affinity for this reagent than any other amino acids. Furthermore, of the two, histidine must be the one more readily able to combine with the diazo acid, as is proved above.

Hence the golden yellow colouration in the histidine reaction can be obtained even in the presence of many other amino acids without their interfering with the result, provided that only a small quantity of the diazobenzenesulphonic acid just sufficient to combine with the histidine be used. If, however, the diazo acid be added in amount more than equivalent to the quantity of histidine an impure mixed brown or reddish yellow colouration is obtained in the secondary reaction owing to the presence of tyrosine or other amino acids. Hydrogen peroxide may then be added to the solution to obtain the pure lemon yellow, characteristic of histidine, the reddish shade being removed by oxidation.

For the detection of histidine in a protein molecule one of the above mentioned methods can be employed. Although the test works well without the necessity of previously hydrolysing the protein, it is nevertheless better to do so.

Two grams of caseinogen were boiled with 6 cc. of concentrated hydrochloric acid under a reflux condenser for seven hours. The solution, after cooling, was almost neutralised by caustic soda solution and then made alkaline with sodium carbonate solution. It was then diluted with water to 100 cc. The following diazo reactions were carried out with 5 cc. of this solution using N/100 diazobenzenesulphonic acid as reagent.

2 % Hydrolysed caseinogen in cc.	5	5	5	5	5	5
N/100 Diazobenzenesulphonic acid in cc.	1	2	3	4	6	10
Primary colouration	brown	brown	brown	dark brown	dark brown	dark red brown
Secondary colouration	pale yellow	yellow	golden yellow	golden yellow with reddish tint	brown yellow	brown yellow
Colouration after oxidation with 20 % hydrogen peroxide	pale lemon yellow	lemon yellow	lemon yellow	lemon yellow	lemon yellow	lemon yellow

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This experiment shows that when the diazo reagent is used in small quantity the secondary colouration is yellow owing to the presence of histidine: as the amount of the reagent increases, the formation of the reddish colour commences and this mixes with the yellow to yield a brown. This phenomenon may be explained by the different degrees of affinity of the amino acids for the diazo reagent, as discussed above. If the pure golden yellow end colouration for histidine be masked by being accompanied by the reddish colouration brought about chiefly by tyrosine, the oxidation process by means of hydrogen peroxide may be used in conjunction with the test, in order to get rid of the interfering tints. In this case a pure lemon yellow colouration of strong intensity indicates the presence of histidine.

I propose to endeavour to apply this modified diazo reaction to the quantitative estimation of histidine in the protein molecule.

Summary.

1. It is easy to distinguish between tyrosine and histidine by means of the diazo reaction by making use of the different colourations produced by reducing their similarly coloured diazo compounds with zinc dust in hydrochloric acid solution, subsequently rendering the solution alkaline with excess of ammonia: tyrosine gives a rose red, histidine a golden yellow colouration.

2. The golden yellow colouration produced by histidine is specific for this amino acid among the protein cleavage products. The reaction may be carried out even with a solution of 1 : 100,000, but the peculiarly characteristic colour comes into appearance only with a solution stronger than 1 : 20,000.

3. The rose red colour of tyrosine is not specific for this amino acid, as the same colouration may be produced by some other amino acids, but taking into consideration the fact that the original diazo reaction is given only by tyrosine and histidine, it seems possible to detect the presence of tyrosine by means of these colour reactions. The delicacy of the modified tyrosine reaction is 1 : 10,000. The reaction has sometimes an advantage over that of Millon as in the case when chlorides are present in large amount.

4. The reddish colourations produced by tyrosine or other amino acids in the secondary diazo reaction are readily destroyed by oxidation with

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hydrogen peroxide, resulting in the formation of nearly colourless or faintly rust coloured solutions, while the golden yellow colouration of histidine is little altered, changing only to a lemon yellow.

5. Using a small quantity of diazobenzenesulphonic acid sufficient only to couple with the histidine, or applying the process of oxidation by means of hydrogen peroxide in conjunction with the reduction, the presence of histidine in proteins can be easily detected by reason of the characteristic colouration formed in the secondary diazo reaction.

In conclusion, I wish to express my indebtedness to Professor Hopkins for his valuable advice and criticism and to Mr Foreman for his kindness in supplying me with some of the amino acids used in this investigation.

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XXXIX. THE EFFECT OF ALKALIS ON THE RATES OF OXIDATION AND REDUCTION OF BLOOD.

By TOYOJIRO KATO.

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(*Received August 24th, 1915.*)

The effect of the addition of acids to blood, in altering the rate at which it can be oxidised or reduced under given conditions, has been studied in this laboratory by Mathison [1911] and by Oinuma [1911], whilst some observations of a similar nature on haemoglobin have been made by Barcroft [1914]. Mathison found that acids accelerated the rate of reduction of blood with a stream of inert gas and that their effect was proportional to the alteration of hydrogen ion concentration produced by them in serum. Oinuma found that, in addition to the acceleration of reduction observed by Mathison, carbonic acid rendered reduced blood less rapidly oxidised by a stream of gas containing oxygen.

No similar observations exist concerning the effect of alkalis. I therefore determined to undertake a research on this subject. The results are given in the following paper.

After testing a number of methods, each of which seemed to promise some improvement on that used by Mathison, I returned to his as being the most satisfactory. Briefly this consists in bubbling gas from a gas holder through a given quantity of blood for a given time. The blood is contained in a tube tilted at an angle of about 15° to the horizontal in a water bath of a given temperature. The tube which I have used has the length of 41 cm. measured from the top to the first bend and the bore is 1 cm. In order to

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prevent "frothing," the greatest difficulty presented by this method, a spiral of wire covered with vaseline is inserted into the tube at a convenient distance above the surface of the blood. Sometimes, especially when the concentration of the added alkali is high, the bubbles are not easily broken and the successive films remain unbroken in the tube; thus the streaming of gas is retarded and it is longer in contact with the blood. Accordingly the reduction or oxidation is accelerated at a given rate and duration of bubbling. In order to get uniform results therefore great attention must be paid to this frothing. Slight frothing often makes an experiment useless.

The actual measurement is that of the percentage saturation of the blood with oxygen after gas (either for the purpose of oxidising or reducing the blood) has been passed through for a measured time. The measurements of the percentage saturation have been made with Barcroft's differential blood gas apparatus, the form used being that designed for 1 cc. of blood and described by Barcroft [1914, p. 291]. The shaking of the apparatus has been carried out by the apparatus used by Barcroft for this purpose.

Whilst it is not necessary to go into further detail here as to the apparatus used, I may state the degree of accuracy which it has afforded me. In the present paper there are more than 170 double determinations of the percentage saturation of blood with oxygen. The divergence observed may be tabulated as follows:

Divergence	1 % and under	1–2 %	2–3 %	3–4 %	4–5 %	5–6 %	6–7 %	7–8 %
Number of determinations	43	39	34	18	15	7	3	1

Data given below in the following form:

$$\left.\begin{matrix}42{\cdot}5\\40{\cdot}0\end{matrix}\right\}\ 41{\cdot}3\ \%$$

signify that gas was bubbled through one sample of blood and that two determinations were made of the resulting blood; but where they are given with a double bracket as follows:

$$\left.\begin{matrix}\left.\begin{matrix}(a)\ 18{\cdot}5\\(b)\ 18{\cdot}2\end{matrix}\right\}\ 18{\cdot}4\\ \left.\begin{matrix}(c)\ 20{\cdot}4\\(d)\ 19{\cdot}3\end{matrix}\right\}\ 19{\cdot}9\end{matrix}\right\}\ 19{\cdot}2$$

the meaning is that the blood was divided into two portions. The gas was bubbled through the first, and determinations (*a*) and (*b*) were performed, then in an exactly similar way through the second, (*c*) and (*d*) being the

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determinations obtained from it. The divergence between (*a*) and (*b*) therefore is simply that caused by errors in the measurement of the percentage saturation, that between the figures in the second column (18·4 % and 19·9 %) includes the errors due to inequality in the rate of bubbling and similar causes. There are four sets of determinations of this character given below, in none of which do the figures in the second bracket differ by as much as four per cent. from one another.

The blood used was always from animals recently killed without use of any narcotics or drugs. When the blood is not fresh enough the sensitiveness to alkali suffers to a considerable extent. In the case of oxen and sheep the animals were usually killed at the slaughter house about one or two o'clock, in which case the experiment was finished that day. Occasionally the butcher killed the animals late in the evening; the blood was then kept in ice till the next morning. When rabbit's blood was used, the animal was killed just before the experiment; the blood was always defibrinated by whipping.

The addition of alkali to the blood. The same routine has been adopted throughout for the addition of alkali to the blood; 4·5 cc. of blood have been taken and 0·5 cc. of a solution of the alkali in physiological saline (0·9–1 % NaCl) added, the alkali in the portion added being ten times the strength to which it was desired to bring the blood. Thus in the tables, if the blood is described as having alkali added to the extent of M/100 NaOH, 0·5 cc. of M/10 NaOH in physiological saline was added to 4·5 cc. of blood. For each control experiment ("normal blood"), to 4·5 cc. of blood 0·5 cc. of physiological saline was added.

The reduction of the blood was always carried out with commercial nitrogen, this was never quite free from oxygen, containing just under 0·5–0·8 % of that gas. The blood was always thoroughly shaken up with air before the reduction commenced, thus ridding it of carbonic acid and saturating the haemoglobin with oxygen.

The oxidation of the blood was carried out with gas which consisted of approximately 12 % oxygen and 88 % nitrogen; as there were slight differences in the quantity of oxygen present in different experiments, the oxygen sometimes rising as high as 12·6 %, the analysis is given at the head of the tables.

The oxidation experiments have, of course, to be carried out on previously reduced blood. This reduction was effected by the bubbling of nitrogen through the blood for an adequate time. This proved to be 40–50 minutes

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at 39–40°, the more alkaline the blood the longer did it take for reduction. A few examples will serve to illustrate the complete reduction.

Blood	Alkali added	Duration of bubbling in minutes	Number of bubbles per min.	Temperature C.	Percentage saturation of blood with O_2 after reduction	
Calf	M/200 NaOH	40	60	38°	−1·0 1·3	0·2
Ox	M/50 NaOH	50	72	39°	−2·4 1·2	−0·6
Rabbit	M/50 $NaHCO_3$	50	80	39°–40°	0·8 −1·2	−0·2

The correction necessary for the exchange of gases in physical solution. In theory this correction differs with each sample of blood. In practice we have applied a uniform correction to our whole series of experiments which is sufficiently accurate for the purpose.

To consider the simplest case. Suppose the blood in the tube at the end of the experiment is completely reduced.

(*a*) 1 cc. of blood will have no oxygen in solution; it will take up from the air in the blood gas analysis apparatus $0{\cdot}031 \times 21/100 = 0{\cdot}0065$ cc. oxygen, since the blood absorbs at 15° about 0·031 cc. of oxygen.

(*b*) It has been in contact with nitrogen at about 710 mm. pressure (that of the atmosphere less the pressure of aqueous vapour at 38°). The amount of gas which it can absorb at 710 mm. pressure and 38° is 0·0103. In the blood gas bottle it is in equilibrium with nitrogen at approximately 600 mm. pressure and at a temperature of about 15°. The amount of nitrogen, which 1 cc. of blood absorbs at 600 mm. pressure and 15° is 0·013. Therefore on shaking up in the blood gas apparatus it gains $0{\cdot}013 - 0{\cdot}0103 = 0{\cdot}00207$ cc. nitrogen. Thus the total amount of gas physically taken up by the blood in the blood gas bottle is $0{\cdot}0065 + 0{\cdot}002 = 0{\cdot}0085$ cc. If the oxygen capacity of 1 cc. blood (diluted with 1/10 of its volume of fluid) be 0·16 cc., then the gas taken up is about 5 % of the oxygen capacity, which must be added as a correction to the observed value of percentage saturation. A similar correction should be made to each experiment for the physical gas exchange.

The Blood Gas of Different Animals.

The blood of different species.

It is clear that the blood of different species of animals presents very different properties both in relation to oxidation and reduction [Barcroft and King, 1909, man and rabbit; Barcroft and Camis, 1909, man and dog].

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That some such difference must exist is the necessary corollary to the fact that when in equilibrium with gas of a uniform partial pressure of oxygen the blood of different species exhibits different percentage saturations. Three animals have been studied in this respect, the ox, the sheep and the rabbit. Of these the properties of ox blood and sheep's blood are very similar, but they differ markedly from rabbit's blood which is on the one hand more readily reduced and on the other less readily oxidised. These facts are shown in the following tables:

SERIES 1. *Comparison of ox and sheep's blood, oxidised with* N_2 (87·4 %) + O_2 (12·6 %).

Animal	Duration of bubbling in minutes	Number of bubbles per minute	Temperature C.	Percentage saturation	
Ox	5	60	39	50·2 52·4	51·3
Sheep	5	60	39	53·8 53·2	53·5

SERIES 2. *Comparison of ox and sheep's blood, reduced with* N_2.

Ox	15	60	38	23·5 23·2	23·4
Sheep	15	60	38	26·8 27·6	27·2

SERIES 3. *Comparison of sheep's and rabbit's blood oxidised with* N_2 (88 %) + O_2 (12 %).

Sheep	10	60	38	62·3 60·5	61·4
Rabbit	10	60	38	47·2 45·2	46·2

SERIES 4. *Comparison of sheep's and rabbit's blood, reduced with* N_2.

Sheep	10	60	38	52·2 54·2	53·4
Rabbit	10	60	38	43·0 44·2	43·6

The blood of different animals of the same species.

The fresh blood from different animals of certain species which we have tested presents much the same properties as regards both oxidation and reduction.

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Percentage saturation of blood with O_2 after reduction with N_2.

Series	Animal	Duration of bubbling in minutes	Rate of bubbling per minute	Percentage saturation of blood with O_2 after reduction	
1	ox 1	7½	60	49·7 50·9	49·9
		12	60	32·1 33·0	32·6
		10	(interpolated)		39·8
	ox 2	10	60	39·6 39·7	39·7
2	sheep 1	5	72	60·0 60·7	61·7
	sheep 2	5	72	65·2 62·0	63·6
	sheep 1	10	72	40·8 38·8	39·8
	sheep 2	10	72	42·4 38·6	40·5
	sheep 1	15	72	22·8 22·6	24·1
	sheep 2	15	72	28·8 25·4	27·1
	sheep 1	20	72	16·1 —	16·1
	sheep 2	20	72	19·4 13·2	16·3
	sheep 1	30	72	−0·2	
	sheep 2	30	72	0·6 4·6	2·6
3	rabbit 1	10	60	45·1 44·2	44·6
	rabbit 2	10	60	43·0 44·2	43·6

Percentage saturation of blood with O_2 after oxidation with N_2 (= 88 %) + O_2 (= 12 %).

Series	Animal	Duration of bubbling in minutes	Rate of bubbling per minute	Percentage saturation of blood	
1	ox 1	5	72	54·8 51·4	53·1
	ox 2	5	72	52·4 52·8	52·6
	ox 1	10	72	84·5 85·2	84·8
	ox 2	10	72	82·8 80·2	81·5
	ox 1	15	72	94·3 96·0	95·2
	ox 2	15	72	91·7 91·7	91·7
2	sheep 1	5	72	53·8 53·2	53·5
	sheep 2	5	72	50·2 52·4	51·3

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However there are many exceptions. Not seldom the blood of animals of the same species has shown a definitely different value of oxygen capacity and also great divergence of percentage saturation with regard both to oxidation and to reduction. It is certain that in man there are slight differences in the percentage saturation of the blood which is in equilibrium with gas containing a uniform partial pressure of oxygen [Barcroft, 1914]. Similar differences have been observed in the blood of different mice with reference to carbon monoxide [Douglas, Haldane, J. S., and Haldane, J. B. S., 1912]. There are no data to show whether these differences are sufficiently large to show themselves in experiments similar to those quoted above.

The Influence of Alkali upon the Reduction and Oxidation of Blood.

Series I. Reduction. The first series of experiments which were rather of a preliminary character consisted in reducing blood made alkaline with NaOH always to the extent of M/200 and varying the circumstances under which the reduction took place, namely, the duration and rate of the bubbling and the animal from which the blood was taken. In every case the more alkaline blood was reduced to a less extent than the control blood to which alkali was not added.

(*a*) Ox blood, 60 bubbles of N_2 per min. at 38°:

Blood	Duration of bubbling in minutes	Percentage saturation after reduction	
Normal	7½	49·7	49·9
		50·0	
+M/200 NaOH	7½	61·0	60·1
		59·2	
Normal	12	32·1	32·6
		33·0	
+M/200 NaOH	12	41·1	42·2
		43·3	

(*b*) Sheep's blood, 64 bubbles of N_2 per min. at 38°:

Normal	10	41·5	38·9
		36·3	
+M/200 NaOH	10	45·6	46·2
		46·7	

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(*c*) Ox blood, 60 bubbles of N_2 per min. at 38°:

Blood	Duration of bubbling in minutes	Percentage saturation after reduction		
Normal	10	39·6, 39·8	39·7	
+M/200 NaOH	10	47·5, 49·2	48·4	
Normal	15	23·5, 23·2	23·4	24·2
		25·4, 24·3	24·9	
+M/200 NaOH	15	32·2, 30·0	31·1	32·6
		33·1, 34·8	34·0	

(*d*) Sheep's blood, 60 bubbles of N_2 per min. at 38°:

Normal	15	26·8, 27·6	27·2
+M/200 NaOH	15	35·0, 30·6	32·8

(*e*) Rabbit's blood, 72 bubbles of N_2 per min. at 38°:

Normal	10	21·8, 16·7	19·3
+M/200 NaOH	10	29·6, 26·0	27·8

(*f*) Sheep's blood, 72 bubbles of N_2 per min. at 38°:

Normal	10	40·1, 37·1	38·6	40·3
		39·9, 43·6	41·8	
+M/200 NaOH	10	50·0, 49·1	49·6	49·1
		50·0, 47·2	48·6	

Series II. Oxidation. The second series consists of a corresponding set of experiments not on the reduction but on the oxidation of blood. The result shows that blood made alkaline to the extent of M/200 NaOH is more rapidly oxidised than is normal blood.

The gas used for the oxidation in this series consisted of O_2, 12 % + N_2, 88 %:

(*a*) Ox blood, 60 bubbles per min. at 38°:

Blood	Duration of bubbling in minutes	Percentage saturation at end of oxidation	
Normal	10	80·1, 79·9	80·0
+M/200 NaOH	10	89·1, 88·5	88·8

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(*b*) Rabbit's blood, 72 bubbles per min. at 38°:

Blood	Duration of bubbling in minutes	Percentage saturation at end of oxidation	
Normal	20	91·8 91·3	91·6
+M/200 NaOH	20	94·3 92·8	93·6

(*c*) Rabbit's blood, 48 bubbles per min. at 39°:

Normal	5	43·7 40·1	41·9
+M/200 NaOH	5	54·8 48·0	51·4

(*d*) Sheep's blood, 48 bubbles per min. at 39°:

Normal	5	41·4 36·7	39·1
+M/200 NaOH	5	48·3 43·3	45·8

The above tables give the percentage saturations attained by the alkaline and normal bloods in a given time. This measurement is of much less theoretical interest than the relative times taken to attain a given percentage saturation, since this latter measurement gives the relative velocities of the two reactions. Some idea of this may be derived in the case of reduction from figures given in Series I. Let V_A/V_N be the ratio of the velocities of reduction of the alkaline and normal blood.

In Series I (*a*), the alkaline blood gives the following data:

	Alkaline blood			Normal blood	V_A/V_N
	Observed		Interpolated	Observed	
Time	7·5	12	9·8	7·5	**0·77**
Percentage saturation	60·1	42·2	50	49·9	
	Normal blood			Alkaline blood	V_A/V_N
Time	7·5	12	9·4	12	**0·78**
Percentage Saturation	49·9	32·6	42·2	42·2	

From Series I (*c*),

	Alkaline blood			Normal blood	V_A/V_N
Time	10	15	12·6	10	**0·78**
Percentage Saturation	48·4	32·6	39·7	39·7	
	Normal blood			Alkaline blood	V_A/V_N
Time	10	15	12·0	15·0	**0·79**
Percentage saturation	39·7	24·2	32·6	32·6	

As the second decimal place in the ratio V_A/V_N is probably valueless, we may conclude that the addition of alkali to the extent of M/200 NaOH reduces the velocity of the reduction to 0·75 – 0·8 of its normal value.

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The Course of Reduction and Oxidation of Alkaline Blood.

Reduction. As it seemed desirable to test this result over a much greater range of the whole process of reduction, both in percentage saturation and time, a second series of experiments was performed in which fair curves were obtained representing the course of the reaction from the fifth to the thirtieth minute of the reaction (there is always some uncertainty about the first minute or two as it is not clear that a steady condition has established itself). The results of two experiments are as follows:

Exp. *A*. Sheep's blood. 72 bubbles per minute of nitrogen at 38°:

Time in minutes		5		10		15	
Percentage saturation of blood with O_2 after reduction	Normal	62·7 60·7	61·7	40·8 38·8	39·8	22·8 25·6	24·2
	+ M/200 NaOH	70·4 72·2	71·3	48·9 52·6	50·8	32·0 29·5	30·8

Time in minutes		20		25		30	
Percentage saturation of blood with O_2 after reduction	Normal	16·1 —	16·1	10·9 3·0	7·0	−0·2 —	−0·2
	+ M/200 NaOH	15·8 19·4	17·6	12·7 10·3	11·5	7·2 3·6	5·4

Exp. *B*. Sheep's blood. Ditto:

Time in minutes		5		10		15	
Percentage saturation of blood	Normal	65·2 62·0	63·6	42·4 38·6	40·5	28·8 25·4	27·1
	+ M/200 NaOH	75·0 70·6	72·8	53·7 51·1	52·4	27·8 34·2	31·0

Time in minutes		20		25		30	
Percentage saturation of blood	Normal	19·4 13·2	16·3	—		0·6 4·6	2·6
	+ M/200 NaOH	19·1 18·4	18·8	—		5·8 10·2	8·0

Figure 1 is a graphic representation of the data furnished by the first of the two experiments given above, which are very concordant. From it the following values for V_A/V_N may be obtained:

Percentage saturation	60	50	40	30	20
V_A/V_N	0·73	0·73	0·78	0·84	0·84

It is not quite clear from these figures whether the ratio V_A/V_N remains constant in the strict sense of the term over the whole course of the reaction, but clearly it remains approximately so.

Oxidation. To turn to the corresponding data which have been obtained with regard to the relative velocities of the oxidation process in the normal blood and that made alkaline to the extent of M/200 NaOH, the following data enable us to construct curves for the purpose of comparison (Fig. 2).

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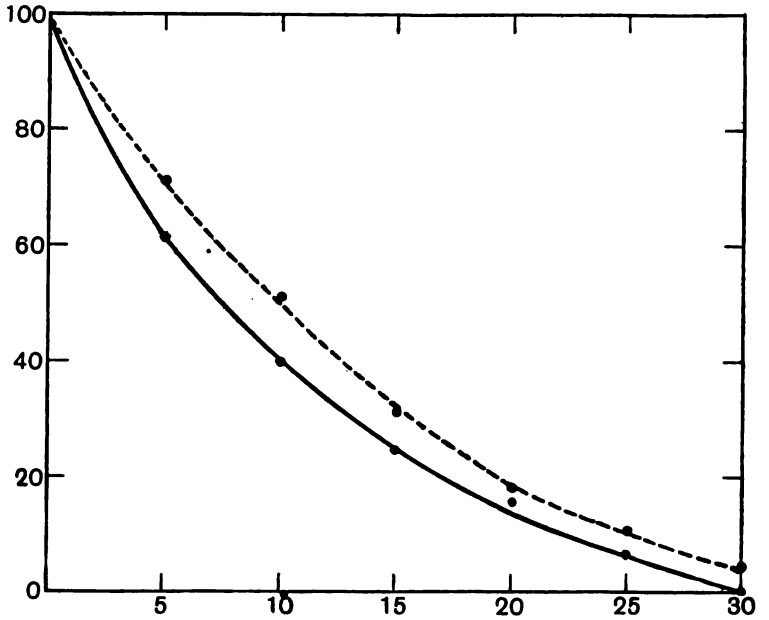

Fig. 1. Rate of reduction of blood with N_2 (Exp. *A*). Ordinates = percentage saturation of blood with O_2. Abscissae = time in minutes. —— normal blood. ..... blood containing M/200 NaOH.

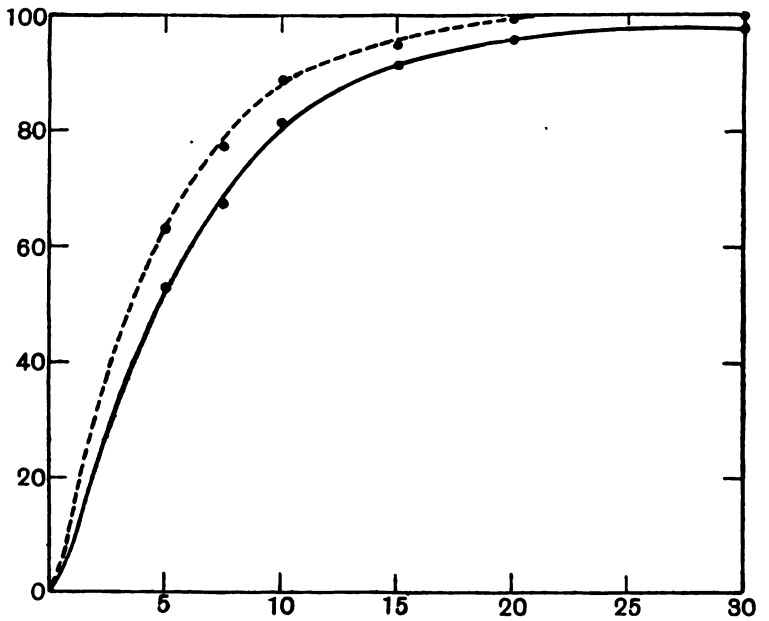

Fig. 2. Rate of oxidation of blood with N_2 (88 %) + O_2 (12 %) (Exp. *A*). Ordinates = percentage saturation of blood with O_2. Abscissae = time in minutes. —— normal blood. ..... blood containing M/200 NaOH.

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Exp. *A*. Ox blood. 72 bubbles of N_2 (88 %) + O_2 (12 %) per min. at 38°:

Time in minutes		5		7½		10	
Percentage saturation of blood	Normal	52·4 52·8	52·6	67·8 66·5	67·2	82·8 80·2	81·5
	+ M/200 NaOH	61·0 63·7	62·4	79·1 75·0	77·1	90·1 87·7	88·9

Time in minutes		15		20		30	
Percentage saturation of blood	Normal	91·7 —	91·7	96·1 96·1	96·1	97·2 98·8	98·0
	+ M/200 NaOH	94·6 95·3	95·0	96·6 102·2	99·4	100·8 —	100·8

The relative velocities of the oxidation process work out from these figures as follows:

Percentage saturation	30	40	50	60	70	80	90
V_A/V_N	1·29	1·29	1·27	1·29	1·29	1·27	1·32

The following data, so far as they go, confirm the value of V_A/V_N obtained above, so far as ox blood is concerned.

Exp. *B*. Ox blood. 72 bubbles of N_2 (88 %) + O_2 (12 %) per min. at 38°:

Time in minutes		5		10		15	
Percentage saturation of blood	Normal	54·8 50·4	53·1	84·5 85·2	84·8	94·3 96·0	95·7
	+ M/200 NaOH	68·3 63·0	65·7	92·6 92·0	92·3	101·0 97·0	99·2

from which may be deduced:

Percentage saturation	60	70	80	90
V_A/V_N	1·30	1·27	1·27	1·28

Taking 1·28 as being the mean value of V_A/V_N for the oxidation process and 0·78 for the reduction process it is worth noting that the one is the reciprocal of the other. That this is not necessarily the case is shown by similar data on the effect of temperature on the oxidation and reduction process [Oinuma, 1911]. Bubbling experiments such as are dealt with in this paper show that the lowering of temperature *greatly* retards the reduction process (i.e., the dissociation of oxygen from haemoglobin) whilst it *scarcely* accelerates the oxidation process, indeed if correction is made for the fact that the oxidation curves at different temperatures reach different final equilibrium points [Oinuma, 1911, Fig. 2], the effect of temperature on the oxidation process becomes negligible.

The following consideration, however, prevents us from attaching too much importance to the reciprocal relation existing between the values of

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V_A/V_N obtained for the oxidation and reduction processes. In the first place one set of experiments was performed on sheep's blood and the other on ox blood. Later it will appear that the bloods of these two species agree closely in their properties, nevertheless it would have been more satisfactory had the two curves been obtained on the same blood. Moreover there seems to be some evidence that V_A/V_N differs in different species. In the rabbit we get a markedly lower value as regards the oxidation process than in the ox:

Exp. *C*. Rabbit's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. at 38°:

Time in minutes		5		10		20	
Percentage saturation of blood	Normal	41·6 —	41·6	65·0 62·5	63·8	86·0 88·1	87·1
	+M/200 NaOH	48·2 47·0	47·6	70·0 72·0	71·0	90·0 89·6	89·8

from which may be deduced:

Percentage saturation	60	70	80
V_A/V_N	1·25	1·19	1·17

Similar experiments on calf's blood gave figures of 1·23–1·26 for calf's blood, slightly below that of ox blood, but on this difference we cannot, without further research, lay any stress.

The Effects of Various Concentrations of Alkali on the Reduction and Oxidation of Blood.

Reduction. So far all the experiments of which I have treated have taken the form of contrasting normal blood with that made alkaline to the extent of M/200 NaOH. I have, however, used both stronger and weaker alkalis than this. Thus the effect of M/400 NaOH is quite appreciable.

The effect of alkali does not seem to be the same at all concentrations. Thus in rabbit's blood NaOH seems to have its maximal effect in retarding the reduction of the blood at some concentration of the order of M/50. With concentrations of NaOH higher than this the blood appears to regain its power of reducing, as is shown by the following table in which the percentage saturation of the blood is given after periods of reduction of 10 and 7·5 minutes respectively.

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The effect of various concentrations of NaOH
on the reduction of blood.

Exp. *A*. Rabbit's blood. 60 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal		+ M/400 NaOH		+ M/200 NaOH		+ M/100 NaOH	
Percentage saturation after reduction	39·7 42·4	41·1	45·0 47·3	46·2	49·5 52·3	50·9	54·8 58·2	56·5
Blood			+ M/50 NaOH		+ M/33 NaOH		+ M/20 NaOH	
Percentage saturation after reduction			66·5 66·8	66·7	55·1 51·3	53·2	48·3 43·1	45·7

Exp. *B*. Rabbit's blood. 60 bubbles of N_2 per min. for 7½ minutes at 38°:

Blood	Normal		+ M/400 NaOH		+ M/200 NaOH	
Percentage saturation after reduction	55·4 56·0	55·7	63·0 61·8	62·4	67·6 66·0	66·8
Blood	+ M/100 NaOH		+ M/50 NaOH		+ M/33 NaOH	
Percentage saturation after reduction	73·0 70·0	71·5	80·4 78·7	79·6	68·9 71·0	70·0

As the spectroscope shows no alkaline haematin in the blood which contains NaOH to the extent of M/20 (for this confirmation I am indebted to Mr Hartridge) this diminished effect of alkali in higher concentration than M/50 is probably due to the laking of blood which occurs to some extent in the course of reduction with this concentration of alkali.

The fact that increased alkali up to M/100 NaOH retards the rate of reduction of blood with nitrogen is shown also for ox blood by the following figures:

Exp. *C*. Ox blood. 60 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal		+ M/200 NaOH		+ M/100 NaOH	
Percentage saturation after reduction	39·6 39·8	39·7	47·5 49·2	48·4	52·7 53·5	53·1

When sodium bicarbonate is used instead of sodium hydroxide, the data are somewhat different as shown in the following table:

The effect of various concentrations of $NaHCO_3$
on the reduction of blood.

Exp. *D*. Sheep's blood. 60 bubbles of N_2 per min. for 10 minutes at 38·5°:

Blood	Normal		+ M/200 $NaHCO_3$		+ M/100 $NaHCO_3$		+ M/33 $NaHCO_3$		+ M/200 NaOH	
Percent. saturation after reduction	53·1 50·2	51·8	54·8 52·5	53·7	57·0 56·1	56·6	59·7 58·5	58·8	60·2 59·2	59·9

Exp. *E*. Rabbit's blood. 60 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal		+ M/200 $NaHCO_3$		+ M/100 $NaHCO_3$		+ M/33 $NaHCO_3$		+ M/20 $NaHCO_3$		+ M/200 NaOH	
Percent. saturation after reduction	45·1 44·2	44·7	49·8 47·1	48·5	52·3 50·0	51·2	63·7 61·3	62·5	68·5 67·7	68·1	56·9 52·4	54·7

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We see now that the retardation of reduction caused by sodium bicarbonate steadily increases with the increased concentration and, unlike the case of sodium hydroxide, also in higher concentration than M/50, even in M/20.

Oxidation. Increased concentration of alkali seems to increase the power of alkali in accelerating the velocity at which reduced blood is oxidised by gas consisting of nitrogen and oxygen in the mixture which I have used. I have worked up to concentrations equal to M/33. This fact is true not only of NaOH but also of $NaHCO_3$ as is shown by the following table:

The effect of various concentrations of alkalis on oxidation of blood.

Exp. *A*. Sheep's blood. 60 bubbles of N_2 (87·4 %) + O_2 (12·6 %) per min. for 5 minutes at 39°:

Blood	Normal		+M/400 NaOH		+M/200 NaOH		+M/100 NaOH		+M/50 NaOH		+M/33 NaOH	
Percent. saturation after oxidation	53·8 53·2	53·5	59·2 56·6	57·9	65·3 61·0	63·2	68·0 63·6	66·8	73·2 74·0	73·6	77·2 73·8	75·5

Exp. *B*. Ox blood. 60 bubbles of N_2 (87·4 %) + O_2 (12·6 %) per min. for 5 minutes at 38°:

Blood	Normal		+M/400 NaOH		+M/200 NaOH		+M/100 NaOH		+M/33 NaOH	
Percent. saturation after oxidation	49·2 52·4	51·3	57·5 54·4	56·0	62·0 57·0	59·5	60·3 61·3	60·8	65·0 71·9	68·5

Exp. *C*. Rabbit's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. for 7½ minutes at 39°:

Blood	Normal		+M/200 $NaHCO_3$		+M/100 $NaHCO_3$		+M/33 $NaHCO_3$		+M/200 Na_2CO_3		+M/200 NaOH	
Percent. saturation after oxidation	54·7 56·5	55·6	59·2 57·6	58·4	61·7 59·7	60·7	67·4 67·5	67·5	62·1 61·3	61·7	66·0 61·2	63·6

Exp. *D*. Sheep's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. for 10 minutes at 39°:

Blood	Normal		+M/200 $NaHCO_3$		+M/100 $NaHCO_3$		+M/50 $NaHCO_3$		+M/200 Na_2CO_3		+M/200 NaOH	
Percent. saturation after oxidation	63·3 61·5	62·4	64·1 65·2	64·7	68·2 66·8	67·5	74·1 72·8	73·5	69·5 68·2	68·8	71·5 69·0	70·3

On adding NaOH to the extent of M/33 there is no fall of the power of alkali to accelerate the rate of oxidation of blood as in the case of reduction. I repeated similar experiments several times but could prove no definite fall of the effects of alkali. Any higher concentration of alkali than M/33 caused intense frothing in the tube towards the end of the preliminary reduction of blood, which must last about 50 minutes or more, and made the experiment useless.

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The Effect of Different Alkalis on the Rate of Reduction and Oxidation of Blood.

I have compared a number of alkaline salts of sodium, under similar conditions. Of these it appears that sodium hydroxide and sodium carbonate in equimolecular proportion do not differ materially in their effects, and that they are more potent both in retarding reduction and in accelerating oxidation than are either dibasic sodium phosphate or sodium bicarbonate, which in turn have approximately equal effects.

Comparison of the effect of different alkalis of equimolecular concentration upon the rate of reduction.

Exp. *A*. Sheep's blood. 72 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal		+ M/200 Na_2HPO_4		+ M/200 $NaHCO_3$		+ M/200 Na_2CO_3		+ M/200 NaOH	
Percent. saturation after reduction	39·5 41·4	40·5	41·2 41·1	41·2	42·6 43·2	42·9	45·8 45·9	45·9	47·8 48·5	48·2

Exp. *B*. Rabbit's blood. 72 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal		+ M/100 Na_2HPO_4		+ M/100 $NaHCO_3$		+ M/100 Na_2CO_3		+ M/100 NaOH	
Percent. saturation after reduction	34·3 33·8	34·1	36·9 36·2	36·6	41·2 39·1	40·2	49·4 50·5	50·0	50·3 49·9	50·1

Comparison of the effect of different alkalis of equimolecular concentration upon the rate of oxidation.

Exp. *C*. Sheep's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. for 10 minutes at 39°:

Blood	Normal		+ M/200 Na_2HPO_4		+ M/200 $NaHCO_3$		+ M/200 Na_2CO_3		+ M/200 NaOH	
Percent. saturation after oxidation	— 69·1	69·1	72·5 70·8	71·7	72·2 71·5	71·9	78·0 77·1	77·6	79·4 77·4	78·4

Exp. *D*. Rabbit's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. for 7½ minutes at 38°:

Blood	Normal		+ M/100 Na_2HPO_4		+ M/100 $NaHCO_3$		+ M/100 Na_2CO_3		+ M/100 NaOH	
Percent. saturation after oxidation	47·4 44·0	46·2	50·0 49·3	49·7	50·1 51·1	50·6	60·0 58·2	59·1	61·3 57·1	59·2

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The Interference of Carbon Dioxide with the Effect of Alkali on the Rate of Oxidation of Blood.

As shown by Bohr, Hasselbalch and Krogh [1907] and also by Mathison [1911] and Oinuma [1911], the presence of carbon dioxide hastens the rate of reduction of blood and slows that of oxidation. We see now that alkali acts in just the opposite way. The last series of the present investigation was undertaken to see the extent to which carbon dioxide and alkali counteract each other with regard to the oxidation of blood. As oxidising gas two mixtures were employed at body temperature: each consisted of nitrogen containing 12·8 % oxygen, but one with 5·6 % CO_2 added, the other without CO_2. The partial pressures of oxygen and carbon dioxide in the former roughly correspond, at body temperature and with the tension of aqueous vapour corrected, to 100 mm. and 40 mm. of mercury respectively; the mixture is therefore approximately equivalent to alveolar air. The following data were obtained after passing a stream of each gas mixture through normal and alkaline blood which had been previously entirely reduced.

Interference of CO_2 *with the effect of alkali upon the rate of oxidation.*

Exp. *A*. Rabbit's blood. 60 bubbles of gas mixture at 39° for 10 minutes:

Gas mixture	Percentage saturation of blood: Normal		+M/100 $NaHCO_3$		+M/100 NaOH	
N_2 (87·2 %) + O_2 (12·8 %)	63·5, 64·0	63·8	75·5, 68·0	69·3	74·7, 72·3	73·5
N_2 (81·6 %) + O_2 (12·8 %) + CO_2 (5·6 %)	56·2, 55·0	55·6	61·9, 58·8	60·9	67·3, 64·0	65·7

Exp. *B*. Rabbit's blood. 60 bubbles of gas mixture at 39° for 10 minutes:

Gas mixture	Percentage saturation of blood: Normal		+M/200 NaOH		+M/100 NaOH	
N_2 (87·2 %) + O_2 (12·8 %)	64·7, 67·0	65·9	73·7, 71·8	72·8	75·1, 77·3	76·2
N_2 (81·6 %) + O_2 (12·8 %) + CO_2 (5·6 %)	57·1, 55·6	56·8	65·8, 63·7	64·8	68·5, 66·3	67·4

From these results it is obvious that blood made alkaline by adding M/100 NaOH is oxygenated to a greater degree by a gas containing 5·6 % CO_2 than normal blood is oxidised by a similar gas which contains no CO_2, and blood with NaOH added to M/200 to a slightly smaller degree. Thus the depressing action of CO_2 at the same partial pressure as in the alveolar air

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on the rate of oxidation of blood is counteracted by the addition of NaOH to the blood to some concentration between M/200 and M/100. The addition of $NaHCO_3$ to M/100 does not wholly counteract the effect of CO_2 of the percentage mentioned above.

If we compare the figures in any one line, we find another fact worthy of mention, i.e., the differences between the percentage saturation of normal blood and that of alkaline blood are practically equal in the cases in which the oxidising gas contained CO_2 and in those in which no CO_2 was contained. Accordingly it may be asserted that the alkali exerts an accelerating effect of about equal extent on the oxidation of blood both in cases where the oxidising gas used contains CO_2 and where no CO_2 is present.

It is clear that these results concerning the effect of alkali on the gas exchange of blood have a certain clinical significance. Numerous observers, Poulton and Ryffel [1913], Peabody [1914], Lewis, Barcroft and their collaborators [1913], have described acid conditions of the blood associated with dyspnoea probably of renal origin. Such conditions might conceivably be treated by the addition of alkali in some form or other to the blood; the line of argument being (1) that the alkali by reducing the hydrogen ion concentration of the blood would relieve the dyspnoea; (2) that it might be added in sufficient quantity to do this in spite of a possible rise in the CO_2 occasioned by the slower respiration. The results given show that the alkali would assist the taking up of oxygen in the lung and would probably not retard the reduction process, in the case of patients resting in bed, sufficiently to impair the respiration in the tissues to an extent which would be really injurious.

Summary.

(1) The rate at which arterial blood gives up its oxygen at low oxygen tensions or the venous blood takes up the gas at high oxygen tensions is influenced to an appreciable degree by the presence of alkali in small quantity. The oxygenation is hastened and the reduction slowed.

(2) The addition of alkali to the extent of M/200 NaOH reduces at body temperature the velocity of reduction of blood on an average to 0·78 of its normal values and increases that of oxidation approximately to 1·2–1·3. Of these two values one is nearly the reciprocal of the other.

(3) The above effect of alkalis (NaOH, $NaHCO_3$) upon the rate of oxidation and reduction increases with increased concentration up to about M/50.

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The power of NaOH in retarding the velocity of reduction appears to fall at a concentration higher than M/50.

(4) In equimolecular concentrations, NaOH and Na_2CO_3 have an equivalent effect. The same is true of Na_2HPO_4 and $NaHCO_3$ but their effect is less than that of NaOH and Na_2CO_3.

(5) In the rabbit the effect of carbon dioxide, which is contained in the oxidising gas in such a proportion as is found in the alveolar air, upon the rate of oxidation of blood is counteracted by the addition of NaOH of concentration between M/200 and M/100.

(6) Blood of animals of different species is reduced or oxidised at different rates. In the same species the rate of reaction is generally similar in different animals. There are, however, many exceptions.

I desire to express my best thanks to Mr Barcroft for his kindly help and advice in this investigation.

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XL. THE PART PLAYED BY THE DIFFERENT BLOOD ELEMENTS IN GLYCOLYSIS.

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When blood is allowed to stand at a suitable temperature an appreciable amount of the sugar present disappears. This glycolysis occurs to a marked extent in human blood; it is also observed in the blood of certain of the lower animals (dog, sheep) [Loeb, 1913], while in other animals (pig) [Melvin, 1912], (goat) [Maclean, 1911], little or no decrease in sugar content appears to take place[1]. This difference explains certain divergent statements encountered in the literature. Not only are there differences in the degree of glycolysis in different animals, but great variations may be observed in the same animal under different circumstances. In certain pathological conditions in man, as will be shown later, glycolysis may be greatly increased.

Various observers have demonstrated that the glycolytic power of blood is essentially dependent on the formed tissue elements, and that the fluid constituent plays no part [Rona and Döblin, 1911; MacLeod, 1913]. In general, the power of destroying sugar has been ascribed to the red corpuscles and various theoretical deductions have been drawn, based chiefly on the normal glucose content of the erythrocytes and the relative inability of glucose to penetrate the envelopes and gain access to their interior [Loeb, 1913]. Rona and Arnheim [1913] attempted to separate the cellular elements of blood into erythrocytes and white cells, and found, on estimating the glycolytic

[1] Possibly, this lack of glycolytic power in certain animals is more apparent than real. The presence of a small amount of some sugar-forming substance such as glycogen might supply sugar to replace that destroyed, so that estimation of the sugar content before and after incubation would give no real clue to the glycolytic activity of the blood.

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activity of the two constituents, that no glycolysis was produced by the white cells while the red cells were distinctly active. Levene and Meyer [1912], however, showed that leucocytes have the power of destroying glucose, at least in the presence of phosphate mixture. Rona and Arnheim then added phosphates to their white cells, and found that this addition played an important part, the white cells now showing marked glycolytic activity.

So far, the evidence pointed to the red cells as the chief agent in the phenomenon in normal blood, but, since the discovery of Levene and Meyer mentioned above, certain observers have assumed, without any real evidence, that glycolysis depends chiefly or entirely on the leucocytes.

The chief obstacles in the way of settling this question were bound up with the difficulty of separating the blood elements into white and red cells; a suitable method for accurately estimating sugar in small amounts of blood was also needed.

In recent years several methods for this purpose have been published but certain of them are unsuitable. One of the best is Bang's micro-method. In the following experiments, a modification of the method described by Gardner and MacLean [1914] has been adopted. The objects aimed at were to avoid filtration of the cuprous oxide, and to increase the readings given by very small amounts of sugar so that very minute differences could be detected and estimated. The method will be fully described in another paper. It is sufficient to mention here, that such a small amount of sugar as 0·1 of a milligram gave a reading of nearly 1 cc. of a standard thiosulphate solution. From this it is obvious that very small differences in sugar content could be easily detected, and that very small amounts of blood sufficed for the determinations. In each experiment 1 cc. blood was used. Contrary to the results of Rona and Arnheim [1913], we find that the leucocytes play a very important role in glycolysis, being very much more active than the red corpuscles; on the other hand our experiments agree with the results of these and other authors who maintain that the red corpuscles are also capable of causing glycolysis. Though the red corpuscles are active in this respect, each red corpuscle is of little importance when compared with a white corpuscle; the very much greater number of red corpuscles in normal blood, however, tends to correct this predominance of the white so that in reality the red cells do account for a good deal of the total glycolysis of blood. In abnormal bloods, such as that of patients suffering from leucocythaemia, the part played by the white cells is very marked as is shown in the following experiment.

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Experiments with Pathological Blood.

Experiment I.

Patient suffering from leukaemia was bled, and to 25 cc. blood 0·1 % potassium oxalate added. The blood was centrifuged and the plasma removed. The upper layer, which contained an excess of leucocytes, was now carefully removed, mixed with plasma, again centrifuged and the upper layer used for glycolysis (*Leucocyte Fraction*). By means of a very fine pipette the lower layers, containing an excess of red cells, were removed mixed with plasma and again centrifuged. The lower layers of this were then taken and used for glycolysis (*Red cell Fraction*).

To each fraction some glucose was added: 1 cc. of each was then taken and the sugar content estimated; both were incubated at 37° for 1·75 hours and the sugar again estimated.

Blood counts of the number of red and white cells in each fraction were made. From the results obtained it is obvious that the fraction containing excess of leucocytes was very much more active than the red cell fraction, which contained but a comparatively small number of leucocytes.

	Before incubation		After incubation			Blood counts	
Blood fraction used	No. of cc. of N/250 thiosulphate used	Sugar present per 100 cc. in g.	No. of cc. of N/250 thiosulphate used	Sugar present per 100 cc. in g.	Sugar destroyed per 100 cc. in g.	Red cells per c.mm.	White cells per c.mm.
Leucocyte fraction	11·75	0·132	4·85	0·054	0·78	6,000,000	231,200
Red cell fraction	12·05	0·134	9·55	0·106	0·28	10,000,000	11,360

Here the activity of the leucocyte fraction was very marked, sugar equivalent to 6·90 cc. N/250 thiosulphate being used up in 1·75 hours, while the red cell fraction, which contained four millions more of cells than the white cell fraction, destroyed sugar only equivalent to 2·50 cc. N/250 thiosulphate.

Part of the original blood was also tested; it contained 3,500,000 red cells and 81,000 white cells per c.mm.

In 1·75 hour it used up sugar equivalent to 3·3 cc. N/250 thiosulphate solution—a greater amount would be found in the case of a normal blood containing five to six million red cells. Similar results to the above were found in other cases. The figures in one more case are given and they will suffice to show that in leukaemic blood the increased leucocyte content plays a marked part in the phenomenon of glycolysis.

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Experiment II.

The blood was divided into the two fractions—white and red cells—as in Experiment I. Incubation here lasted for two hours.

	Before incubation		After incubation			Blood counts	
Blood fraction used	No. of cc. of N/250 thiosulphate used	Sugar present per 100 cc. in g.	No. of cc. of N/250 thiosulphate used	Sugar present per 100 cc. in g.	Sugar destroyed per 100 cc. in g.	Red cells per c.mm.	White cells per c.mm.
Leucocyte fraction	5	0·055	none	none	0·055 +	5,000,000	212,400
Red cell fraction	4·5	0·050	1·55	0·017	0·033	10,000,000	17,640

In this experiment the amount of sugar added was too small and the incubation period rather long, for all the sugar was used up in the leucocyte fraction. The numbers obtained for this fraction are therefore too low, for if more sugar had been present more would have been destroyed. While showing the marked effect of the leucocytes these experiments also prove that the red cells take an active part in glycolysis.

They also show that the addition of phosphates is not necessary to bring about this effect. Since the above experiments were done with pathological blood, it might be argued that no deductions applicable to normal blood could be drawn from the results. Nearly twenty experiments, however, have been carried out on normal blood and in every case it has been found that while both red cells and leucocytes are active, the leucocytes are cell for cell very much more active than the red cells. The exact glycolytic power of the white cell compared with the erythrocyte varies very much indeed, but in a general way the experiments suggest that each white cell is from 200 to 1000 times as active in glycolysis as a red cell. Seeing that many delicate manipulations are necessary in arriving at these results it is probable that no great importance is to be attached to the exact figure found for the red to white ratio in any given experiment; it is merely to be regarded as something of the order of the number given. When, however, it is found that in every experiment the glycolytic value for white cells comes out enormously greater than that for the red cells the part played by the leucocytes is abundantly demonstrated. No doubt the destruction of sugar caused by the red cells may depend on certain conditions already mentioned such as the amount of sugar inside the cell, and on other factors: probably also the activity of the white cell varies very much in different cases. If we assume that the white cell is on an average 1000 times as powerful a glycolyser as a red cell, and that normal blood contains about five hundred times as many red cells as white, the leucocytes in a sample of such a blood would account for the destruction of twice as much sugar as the red cells.

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Experiments with Normal Blood.

We found it quite impossible to separate the blood into two fractions—one containing white cells only, and the other red cells. Invariably our red cell fraction contained leucocytes and our leucocyte fraction had always large amounts of red corpuscles. It is obvious, however, that complete separation into two constituents is not necessary. All that is required in order to determine the relative action of the red and white blood cells on glycolysis is to divide the blood into two fractions in which, though both constituents are present, they differ greatly in number. The sugar in each part is estimated before and after incubation. This at once gives conditions from which the effect of either constituent can be worked out by a simple algebraical equation. The following experiments illustrate these points.

Experiment III.

12 cc. of blood were obtained from a student by inserting a needle in a vein, coagulation being prevented by the addition of 0·1 % potassium oxalate. The blood was centrifuged, the plasma separated and the top layer pipetted off. This layer constituted the leucocyte fraction, while the lower layer represented the red cell fraction. The sugar was estimated in each fraction before and after incubation for 4 hours at 37°.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g. per 100 cc.	0·037	0·040
Sugar after incubation in g. per 100 cc.	0·009	0·006
Total sugar used in g. per 100 cc.	0·028	0·034
Blood count in cells per c.mm.	Red cells 6,000,000 White cells 6,960	Red cells 8,000,000 White cells 4,440

Ratio of activity of leucocyte to red cell = 178 : 1.

Also it is evident that both red and white cells must have been active, for, though the white cell is much more active than the red, if the glycolysis depended only on the white corpuscles, the leucocyte fraction should have destroyed much more total sugar than the red cell fraction.

Experiment IV.

Blood from a patient was treated exactly as in Experiment III with the exception that incubation was restricted to 2 hours at 37°.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g. per 100 cc.	0·033	0·032
Sugar after incubation in g. per 100 cc.	0·010	0·013
Total sugar used in g. per 100 cc.	0·023	0·019
Blood count in cells per c.mm.	Red cells 7,200.000 White cells 15,520	Red cells 9,800,000 White cells 2,240

Ratio of activity of leucocyte to red cell = 364 : 1.

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Five other experiments were carried out in exactly the same way except that in certain of them a small amount of glucose was added. The results are given in the table. In No. 8 the red cell fraction first obtained was again centrifuged and the bottom layer of the latter used as the red cell fraction.

No.	Source of Blood	Amount of blood taken in cc.	Sugar added in mgrs.	Time of incubation in hrs.	Sugar of leucocyte fraction: Percent. before incubation	Sugar of leucocyte fraction: Percent. after incubation	Total sugar destroyed by leucocyte fraction per 100 cc.	Sugar of red cell fraction: Percent. before incubation	Sugar of red cell fraction: Percent. after incubation	Total sugar destroyed by red cell fraction per 100 cc.
V	student	15	none	1·5	0·03	0·009	**0·021**	0·028	0·01	**0·018**
VI	patient	15	15	2	0·091	0·061	**0·030**	0·083	0·058	**0·025**
VII	student	20	15	3	0·097	0·056	**0·041**	0·088	0·052	**0·036**
VIII	patient	15	10	1·75	0·084	0·062	**0·022**	0·072	0·058	**0·014**
IX	student	15	10	—	0·062	0·031	**0·031**	0·063	0·049	**0·014**

The "blood counts" of the above experiments gave the figures per cubic millimetre.

	Leucocyte fraction	Red cell fraction
No. V	Red cells 9,200,000 White cells 10,440	Red cells 12,500,000 White cells 3,600
No. VI	Red cells 4,700,000 White cells 13,040	Red cells 10,700,000 White cells 3,840
No. VII	Red cells 4,500,000 White cells 12,480	Red cells 10,000,000 White cells 3,840
No. VIII	Red cells 8,200,000 White cells 23,000	Red cells 10,000,000 White cells 1,280
No. IX	Red cells 5,000,000 White cells 29,320	Red cells 11,800,000 White cells 1,000

	Ratio of leucocyte activity to red cell activity
No. V	863 : 1
VI	965 : 1
VII	850 : 1
VIII	358 : 1
IX	779 : 1

It is obvious from these experiments that the leucocyte is in every case much more active than the red cell in the production of glycolysis.

Glycolysis in Haemolysed Blood.

If, according to the observations of Loeb and others, the power of destroying sugar resided entirely in the red cell it is difficult to understand the results obtained by Edelmann [1912], who showed that laking of the red cells by saponin does not destroy the glycolytic power. It is known, however, that it is possible under certain conditions to lake the red cells without destroying the leucocytes, and it may possibly be that in these experiments where the red corpuscles were destroyed, the white corpuscles were left sufficiently active partially to destroy the sugar present.

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In all the experiments already given, the blood was centrifuged in its own plasma. In some later experiments, some small differences in manipulation were introduced and the corpuscles were washed with isotonic saline solution. Here the results were in agreement with those obtained with unwashed corpuscles except that the ratio of leucocyte activity to that of the red cell was somewhat changed. In all cases clotting was prevented by the addition of 0·1 % potassium oxalate solution.

Experiment X.

The blood was obtained from a student in the usual manner. To this 20 mgrs. sugar were added; after centrifuging, the plasma was removed and the top layer of the solid separated; this was shaken up with the separated plasma and again centrifuged. The top layer of this was mixed with normal saline, again centrifuged and the top layer again pipetted off and used for the experiment (= leucocyte fraction). After the first centrifuging of the blood the layer left behind after removal of the leucocytes was mixed with some normal saline. This was then centrifuged, the lower part of the solid layer formed removed by a fine pipette, again mixed with normal saline, and centrifuged. The lower layer of this, which contained only a relatively small number of white cells, represented the red cell fraction. Incubated 1·5 hours.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g. per 100 cc.	0·0810	0·0792
Sugar after incubation in g. per 100 cc.	0·0468	0·0567
Total sugar destroyed in g. per 100 cc.	0·0342	0·0225
Blood count in cells per c.mm.	Red cells 7,725,000 White cells 23,520	Red cells 8,275,000 White cells 840

Ratio of activity of white to red cell = 218 : 1.

Experiment XI.

This was carried out exactly as in Experiment X. The blood was obtained from a healthy soldier. To 20 cc. blood, 20 mgrs. sugar were added.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g. per 100 cc.	0·091	0·078
Sugar after incubation in g. per 100 cc.	0·052	0·062
Sugar destroyed in g. per 100 cc.	0·039	0·016
Blood count in cells per c.mm.	Red cells 8,225,000 White cells 42,160	Red cells 8,625,000 White cells 1,120

Ratio of activity of white to red cell = 325 : 1.

In these experiments it will be noticed that the activity of the white corpuscle compared with the red cell is low. This however is apparently not due to the action of the saline, as the next experiment shows.

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Experiment XII.

20 cc. blood from student treated exactly as above. Incubation 1·75 hours. 20 mgrs. sugar added.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g per 100 cc.	0·138	0·091
Sugar after incubation in g. per 100 cc.	0·110	0·078
Sugar destroyed in g. per 100 cc.	0·028	0·013
Blood count in cells per c.mm.	Red cells 6,000,000 White cells 17,600	Red cells 10,000,000 White cells 1,800

Ratio of activity of white to red cell = 1149 : 1.

Several experiments were carried out and always with the same general result, though the figures obtained varied considerably. The results given serve to establish the activity of both red and white corpuscles in the process of glycolysis.

Conclusions.

(1) Both the red and white cells of normal blood take part in glycolysis.

(2) The white cells are much more active than the red; the ratio of activity of the white to the red cell varies roughly from about 200:1 up to 1000:1.

(3) In leukaemic or other blood containing excess of leucocytes, glycolysis is much more active than in normal blood.

(4) In normal blood it is probable that the comparatively small number of leucocytes present exert a somewhat greater influence on the total glycolysis than do the red cells which are present in relatively large amount.

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XLI. THE INFLUENCE OF FAT AND CARBOHYDRATE ON THE EXCRETION OF ENDOGENOUS PURINES IN THE URINE OF DOG AND MAN.

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PART I.

In the year 1897 Camerer [1897] showed for the first time that there are two sources of origin for the uric acid in the urine of mammals, viz. (1) the nucleins of the tissues which are destroyed in the course of intracellular metabolism, and (2) the nucleins contained in food. In 1900 Burian and Schur [1900] distinguished these two sources of urinary purines as endogenous and exogenous. As regards the endogenous moiety of the urinary purines, Burian and Schur came to the conclusion that under normal conditions, when the calorie value of the purine-free diet is sufficient, the output of purine is quite independent of the total amount of nitrogen excreted and further that it remains a constant for each individual, although the range of variation in different individuals may be fairly wide. They believed however that the endogenous purine output might be changed by abnormal conditions. About the same time but independently Siven [1901] came to practically a similar conclusion. It is worthy of note that even in 1888 when the genetic relation between the purine compounds and uric acid was not fully appreciated Marĕs [1888] had drawn attention to the constancy of the individual excretion of uric acid and to the fact that the amount excreted varied in different individuals. This constancy in the output of endogenous purines was confirmed by Schreiber and Waldvogel [1899], Rockwood [1904], Macleod

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and Haskins [1906], Walker Hall [1904], Kaufmann and Mohr [1902] and Pfeil [1903].

Folin [1905] from his experiments, in which he used two diets, both purine-free, but one rich in protein and the other poor, came to the conclusion that the uric acid output is to a certain extent dependent on the protein content of the diet; with a reduction in the nitrogen excretion there is a corresponding reduction in the output of uric acid. Smetanka [1911] found an increased output of uric acid after the ingestion of protein and of carbohydrate, and Rosenfeld and Orgler [1896] noted that the output of uric acid was much increased when fat or carbohydrate was added to a diet consisting wholly of meat.

Cathcart [1907] observed that during fasting there was at first a fall in the excretion of uric acid but after a few days it gradually increased until it equalled the original amount. In another series of experiments this worker [1909] found that when a subject was put on a purine-free, carbohydrate-rich diet the uric acid excretion was remarkably high whereas when the diet was changed to one in which fat predominated the excretion of uric acid fell to a very low figure. Recently Graham and Poulton [1913] published a paper in which they described the influence of fats and carbohydrates on the endogenous purine output. They also observed that a fat-rich diet was associated with a low and a carbohydrate-rich diet with a high purine output. They ascribed the high output on the carbohydrate diet to a synthesis of purine from the carbohydrate and protein.

The present experiments were carried out in an attempt to get further light on the possibility of a synthesis of uric acid in the mammalian body. They were performed on the writer himself, 31 years of age, 65 kilos in weight and perfectly healthy. During the course of the experiments all excessive exercise was avoided. The diet consisted of purine-free food-stuffs and was consumed in three portions. The amount of fluid consumed was not measured but was approximately constant. Olive oil had to be substituted after the first diet for butter and cream as these food materials could not be taken by the subject on account of the diarrhoea which they induced.

The analytical methods employed were as follows: total nitrogen, Kjeldahl; ammonia, Folin; uric acid, Hopkins' method as described by Kennaway [1909]; purine bases, Camerer-Arnstein, after removal of uric acid according to Kennaway; acetone and acetoacetic acid by Messinger-Huppert. All the figures recorded are the means of closely agreeing duplicates.

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Experiment I.

Diets consumed in different periods.

I *A*		I *B*		I *C*	
Dried skimmed milk	= 46 g.	Dried skimmed milk	= 105 g.	Dried skimmed milk	= 46 g.
Double cream	= 20 „	Water biscuit	= 100 „	Water biscuit	= 66 „
Butter	= 24 „	Olive oil	= 164 „	Caseinogen	= 27 „
Tapioca	= 117 „	NaCl	= 5 „	Olive oil	= 190 „
Water biscuit	= 341 „			NaCl	= 5 „
Cane sugar	= 20 „				
NaCl	= 5 „				
Protein	= 50 g.	Protein	= 50 g.	Protein	= 50 g.
Carbohydrate	= 443 „	Carbohydrate	= 137 „	Carbohydrate	= 80 „
Fat	= 30 „	Fat	= 165 „	Fat	= 190 „
Calorie intake	= 2300 cal.	Calorie intake	= 2300 cal.	Calorie intake	= 2300 cal.

In the three periods of this experiment the protein intake and calorie value were kept approximately constant whilst the carbohydrate and fat content was varied. Period *A* was carbohydrate-rich, fat-poor; in *B* carbohydrate was largely, and in *C* almost entirely, replaced by fat. Between each period of the experiment a few days elapsed. The first day's urine of each period in this and in all subsequent experiments was discarded. The results of these diets on the composition of the urine are found in Tables I *A*, I *B*, and I *C*.

TABLE I *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
I	Acidic	7·53	0·46	0·110	0·008	0·118	6·11	1·46	0·11	1·57
II	„	8·26	0·46	0·108	0·012	0·120	5·57	1·31	0·15	1·46
III	„	7·79	0·43	0·093	0·011	0·104	5·52	1·19	0·14	1·33
IV	„	7·63	0·39	0·092	0·014	0·106	5·11	1·21	0·18	1·39
V	„	7·99	0·41	0·095	0·013	0·108	5·13	1·19	0·16	1·35
VI	„	7·63	0·39	0·089	0·016	0·105	5·11	1·17	0·21	1·38
Average		(7·81)	(0·42)	(0·098)	(0·012)	(0·110)	(5·43)	(1·26)	(0·16)	(1·41)

TABLE I *B*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
I	Acidic	7·71	0·45	0·095	0·012	0·106	5·84	1·23	0·16	1·39
II	„	7·56	0·37	0·087	0·018	0·105	4·89	1·15	0·24	1·39
III	Amphoteric	8·11	0·36	0·090	0·019	0·109	4·44	1·11	0·23	1·34
IV	„	8·50	0·29	0·095	0·014	0·109	3·41	1·12	0·16	1·28
V	„	8·60	0·25	0·097	0·018	0·115	2·91	1·13	0·21	1·34
VI	„	8·55	0·29	0·090	0·019	0·109	3·39	1·05	0·22	1·27
Average		(8·17)	(0·34)	(0·092)	(0·017)	(0·109)	(4·15)	(1·13)	(0·20)	(1·34)

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TABLE I *C*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
I	Acidic	9·86	0·76	0·074	0·024	0·098	7·71	0·75	0·24	0·99
II	„	7·90	0·71	0·070	0·018	0·088	8·99	0·89	0·23	1·12
III	„	8·51	0·76	0·078	0·021	0·099	8·93	0·92	0·25	1·17
IV	„	8·32	0·67	0·071	0·017	0·088	8·05	0·85	0·20	1·05
V	„	9·25	0·96	0·057	0·024	0·081	10·38	0·62	0·26	0·88
Average		(8·77)	(0·77)	(0·070)	(0·021)	(0·091)	(8·81)	(0·81)	(0·24)	(1·04)

It will be noted that the reaction of the urine in period *B* is amphoteric from the third day and in this way differs from the urine of the first and third periods. This variation in reaction is almost certainly due to the fact that the diet of this period contained a large amount of dried skimmed milk, rich in alkaline salts. This was added to bring the protein content up to the proper level.

Total nitrogen. These three experiments demonstrate clearly the well-marked protein sparing action which the carbohydrates possess. In the carbohydrate-rich period *A*, the average nitrogen excretion is 7·81 g., in the second period it has increased slightly to 8·17 g. and in the fat-rich third period it stands at 8·77 g.

Ammonia. It is a well-known fact that if carbohydrate be taken in insufficient amount, particularly when the diet is rich in fat, acidosis results and this was of course found in this experiment. The reactions for acetone and acetoacetic acid were pronounced in the third period, but were completely absent in the first. Under these conditions it was to be expected that the output of ammonia would be lowest in period *A* and highest in *C* and be intermediate in amount in period *B*. As a matter of fact the average output in the first period was 0·42 g. N as ammonia, it fell to 0·34 g. in the second period and rose to 0·77 g. in the third. The explanation of the abnormal second period lies in the fact already mentioned that in period *B* dried skimmed milk was present in large amounts and the alkaline salts prevented any undue call on ammonia for neutralisation purposes.

Uric acid. In the first period there was a gradual fall in the output of uric acid during the course of the experiment from 0·11 g. on the first day to 0·089 g. on the sixth day, giving an average output of 0·098 g. In the second period the output was less varied, the average for the period being 0·092 g. In the third period the maximum output was 0·078 g. on the third day and the minimum 0·057 g. on the fifth day, giving an average of 0·070 g.

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Thus it is found that the greatest excretion of uric acid was during the carbohydrate-rich first period and the lowest with the fat-rich diet. This effect is also clearly seen when the percentage figures are considered.

There is however one point which deserves some attention, namely that the output during the second period differs hardly at all from that during the first in spite of the great differences in carbohydrate content of the diet. This again must be ascribed to the presence of the alkali-rich dried milk and in support of such a contention there is the work of Macleod and Haskins [1906] who showed that the output of endogenous uric acid is increased by the ingestion of alkali. This point will be dealt with in another experiment in this paper. (Experiment III.)

Purine bases. The output of these bases in all three periods is not very constant. It is interesting to note that in the first period, in contradistinction to the uric acid, there is a gradual increase in the output of the basal purines. The average output for period *A* was 0·012 g. N as purine bases. In the other two periods there is no evidence of a progressive fall, the average excretion for the second period was 0·017 g. and for the third 0·021 g.

It will be observed that the purine base output reaches its lowest on the carbohydrate-rich first diet whilst the figure for the second period is about the mean value of the other two. This result agrees with the observation of Graham and Poulton who found that when carbohydrate was diminished in a diet, the purine base output showed a tendency to rise whilst the uric acid diminished.

The average values of the total purine output for the three periods were *A* 0·110 g., *B* 0·109 g. and *C* 0·091 g. N.

Experiment II.

In this experiment the effect of varying either the content of fat (Period *A*) or of carbohydrate (Period *B*) was tried, the content of protein and of carbohydrate or fat respectively being kept constant. As this variation necessitated a large variation in the calorie value of the diet, the duration of the individual periods of observation was reduced to two days so as to prevent invalidation of the experiment due to insufficient energy supply.

The total nitrogen and ammonia figures show nothing of note. As regards the uric acid the average value in the first two days was 0·068 g. and in the second 0·092 g. From these figures it is clear that when fat was withdrawn in large amount, notwithstanding the diminution in the energy

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value of the diet, the uric acid output both in absolute amount and in percentage value of total nitrogen rose. This rather suggests that fat may have some inhibiting action on the uric acid output.

As regards the purine bases there is no appreciable difference between the outputs under the altered conditions.

Period A.

Diet during first two days		Diet during second two days	
Water biscuit	60 g.	Water biscuit	60 g.
Milk	500 cc.	Milk	500 cc.
Boiled egg white	120 g.	Boiled egg white ...	120 g.
Boiled egg yolk	80 „	Boiled egg yolk	80 „
Olive oil	147 „	NaCl	5 „
NaCl	5 „		
Protein	52 g.	Protein	52 g.
Carbohydrate	79 „	Carbohydrate	79 „
Fat	190 „	Fat	43 „
Calorie intake	2304 cal.	Calorie intake	937 cal.

TABLE II *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Test for aceto-acetic acid
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines	
I	Acidic	9·97	0·74	0·068	0·019	0·087	7·42	0·68	0·19	0·87	+
II	„	9·88	0·77	0·067	0·017	0·084	7·79	0·67	0·17	0·84	+
Average		(9·93)	(0·76)	(0·068)	(0·018)	(0·086)	(7·61)	(0·68)	(0·18)	(0·86)	
III	„	9·80	0·51	0·087	0·018	0·105	6·14	0·89	0·18	1·07	–
IV	„	10·30	0·54	0·097	0·016	0·113	5·24	0·94	0·16	1·10	–
Average		(10·05)	(0·53)	(0·092)	(0·017)	(0·109)	(5·69)	(0·92)	(0·17)	(1·09)	

In this series the protein and fat were kept constant but there was a variation of 259 g. of carbohydrate. Here the uric acid output fell with the diminution in the carbohydrate content from 0·111 g. to 0·096 g. N, a fact which is even more clearly demonstrated when the percentage figures are considered.

The result of both sets of experiments in *A* and *B* quite agree with the observation of Cathcart who found that the administration of fat after a period of fasting reduces the output of uric acid, whilst the administration of carbohydrate increases the output.

These observations are certainly very suggestive of a synthesis of uric acid in the organism as suggested by Wiener [1902] from lactic acid and urea. The urea is always present in considerable amount and lactic acid

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is certainly one of the products which result from the catabolism of carbohydrate.

Period B.

Diet consumed during first two days		Diet consumed during second two days	
Water biscuit	160 g.	Water biscuit	100 g.
Milk	500 cc.	Milk	415 cc.
Rice	412 g.	Boiled egg white ...	120 g.
Cane sugar	36 „	Caseinogen	12 „
NaCl	5 „	NaCl	5 „
Protein	52 g.	Protein	52 g.
Carbohydrate	368 „	Carbohydrate	109 „
Fat	15 „	Fat	15 „
Calorie intake	1862 cal.	Calorie intake	800 cal.

TABLE II *B*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Test for aceto-acetic acid
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines	
I	Acidic	8·24	0·59	0·117	0·012	0·129	7·16	1·42	0·15	1·57	–
II	„	7·28	0·61	0·105	0·017	0·122	8·38	1·44	0·23	1·67	–
Average		(7·76)	(0·60)	(0·111)	(0·015)	(0·126)	(7·77)	(1·43)	(0·19)	(1·62)	
III	Amphoteric	8·20	0·45	0·100	0·017	0·117	5·49	1·22	0·21	1·43	–
IV	Acidic	9·25	0·47	0·091	0·014	0·105	5·08	0·98	0·15	1·13	–
Average		(8·73)	(0·46)	(0·096)	(0·016)	(0·111)	(5·29)	(1·10)	(0·18)	(1·28)	

Another experiment (II *C*) was carried out when the alteration in the carbohydrate content was made in a diet with a high intake of fat, and compared with the first set of figures of II *A*.

Diet during two days of Period *C*		Diet as in the first series of Period II *A*	
Milk	415 cc.		
Water biscuit	160 g.		
Rice	412 „		
Cane sugar	36 „		
Olive oil	175 „		
NaCl	5 „		
Protein	52 g.	Protein	52 g.
Carbohydrate	368 „	Carbohydrate	79 „
Fat	190 „	Fat	190 „
Calorie intake	3489 cal.	Calorie intake	2304 cal.

TABLE II *C*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Test for aceto acetic acid
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines	
I	Acidic	8·06	0·48	0·108	0·011	0·119	5·96	1·34	0·14	1·48	–
II	„	7·52	0·63	0·118	0·009	0·127	8·36	1·57	0·12	1·69	–
Average		(7·79)	(0·56)	(0·113)	(0·010)	(0·123)	(7·17)	(1·46)	(0·13)	(1·59)	

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TABLE II *D*.

	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
	Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
Average values in the first series of Table II *A*	9·93	0·76	0·068	0·018	0·086	7·61	0·68	0·18	0·86
Average values of Table II *C*	7·79	0·56	0·113	0·010	0·123	7·17	1·46	0·13	1·59

It will be observed that there is a very decided difference between the total nitrogen output in the two sets of figures, this being much diminished on the carbohydrate-rich diet. There is also much less ammonia excreted with this diet. Again the uric acid output rises definitely, with the increase in the carbohydrate, from 0·068 g. to 0·113 g. N. This large rise, much more definite than in II *B*, is probably due to a synthesis but it may be also in part due to the disappearance of the acidosis.

There is also a marked difference between the output of the purine bases. Again it is found that the excretion of the bases is higher on the carbohydrate-poor than on the carbohydrate-rich diet.

Another interesting comparison may be made between the first series of II *B* and II *C* where the diets contained the same amounts of protein and carbohydrate but varied in their fat content.

Diet as in the first series of II *B*		Diet during II *C*	
Protein	52 g.	Protein	52 g.
Carbohydrate	368 „	Carbohydrate	368 „
Fat	15 „	Fat	190 „
Calorie intake	1862 cal.	Calorie intake	3489 cal.

TABLE II *E*.

	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
	Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
Average values in the first series of Table II *B*	7·76	0·60	0·111	0·015	0·126	7·77	1·43	0·19	1·62
Average values of Table II *C*	7·79	0·56	0·113	0·010	0·123	7·17	1·46	0·13	1·59

As will be seen the alteration in the fat content of a diet rich in carbohydrate affects but little the composition of the urine except as regards the purine bases.

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Experiment III.

This experiment was carried out for the purpose of testing the influence of alkali on the output of uric acid and purine bases on a fat-rich carbo hydrate-poor diet and on a fat-poor carbohydrate-rich one, the protein content and calorie value of the diets being kept approximately constant.

III *A*. Fat diet		III *B*. Carbohydrate diet	
Water biscuit	63 g.	Milk	500 cc.
Milk	500 cc.	Boiled egg white ...	120 g.
Boiled egg white ...	120 g.	Boiled egg yolk	80 „
Boiled egg yolk	80 „	Cane sugar	214 „
Olive oil	147 „	Rice	120 „
NaCl	5 „	Tapioca	160 „
		NaCl	5 „
Protein	52 g.	Protein	52 g.
Carbohydrate	79 „	Carbohydrate	413 „
Fat	190 „	Fat	43 „
Calorie intake	2304 cal.	Calorie intake	2306 cal.

TABLE III *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Aceto-acetic acid g. reckoned as acetone	Test for aceto-acetic acid	Remarks
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines			
I	Acidic	9·04	0·59	0·091	0·016	0·107	6·53	1·01	0·18	1·18	?	+	
II	„	8·40	0·62	0·070	0·016	0·086	7·38	0·83	0·19	1·02	0·103	+	
III	„	10·47	0·63	0·075	0·014	0·089	6·02	0·72	0·13	0·85	0·237	+	
IV	Alkaline	9·41	0·24	0·086	0·026	0·112	2·55	0·91	0·28	1·19	0·297	+	15 g. $NaHCO_3$ taken
V	„	9·41	0·12	0·121	0·017	0·138	1·28	1·29	0·18	1·47	0·305	+ +	
VI	„	8·99	0·16	0·099	0·017	0·116	1·78	1·10	0·19	1·28	0·395	+ +	
VII	Amphoteric	8·52	0·33	0·085	0·025	0·110	3·47	0·89	0·26	1·16	0·524	+ +	
VIII	Acidic	9·36	0·56	0·079	0·018	0·097	5·98	0·84	0·19	1·04	0·432	+ +	
IX	„	9·84	0·74	0·074	0·017	0·091	7·52	0·75	0·17	0·92	0·515	+ +	

TABLE III *B*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Aceto-acetic acid g. reckoned as acetone	Test for aceto-acetic acid	Remarks
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines			
I	Acidic	7·76	0·50	0·111	0·020	0·131	6·44	1·43	0·26	1·69	0	–	
II	„	7·22	0·52	0·095	0·021	0·116	7·20	1·32	0·29	1·61	0	–	
III	„	6·89	0·52	0·100	0·018	0·118	7·69	1·45	0·26	1·71	0	–	
IV	Alkaline	6·99	0·20	0·111	0·019	0·130	2·86	1·59	0·27	1·86	0	–	15 g. $NaHCO_3$ taken
V	„	6·01	0·09	0·113	0·012	0·125	1·56	1·88	0·20	2·08	0	–	
VI	„	6·14	0·09	0·121	0·016	0·137	1·47	1·97	0·26	2·23	0	–	
VII	Amphoteric	6·08	0·28	0·096	0·018	0·114	4·61	1·58	0·30	1·88	0	–	
VIII	Acidic	6·28	0·46	0·096	0·022	0·118	7·35	1·53	0·35	1·88	0	–	
IX	„	6·36	0·46	0·089	0·020	0·109	7·23	1·40	0·31	1·71	0	–	

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In each series of experiments for three days (fourth to sixth day) 5 g. of sodium bicarbonate were taken thrice daily so that the urine was kept alkaline for this period.

Total nitrogen. There is no evidence of any marked influence of the alkali.

Ammonia. The administration of the bicarbonate causes a very definite fall in the amount of ammonia excreted. The average output on the non-alkali days in the case of the fat diet was 0·58 g. N and on the alkali days 0·17 g. N, in the case of the carbohydrate it fell from 0·46 g. to 0·13 g.

Uric acid. Haig [1887], Gorsky [1888] and Macleod and Haskins [1906] have all observed an increased output of uric acid after the administration of alkali. In the present series the variation between the two averages of uric acid output on the fat and carbohydrate diets, excluding the alkali days, is very definite: 0·077 g. N with the fat and 0·096 g. with the carbohydrate (average of five days omitting the first day in each experiment). As the result of giving bicarbonate the uric acid excretion rose in both sets of experiments; on the fat diet the average value for the three days was 0·102 g. N, an increase of 25 mg. over the output on non-alkali days, and on the carbohydrate diet the average value for the three days was 0·115 g., i.e. 19 mg. higher than the average output on the non-alkali days. Thus it would appear that the administration of an alkali does definitely increase the output of uric acid and it would further seem to be more effective in the case of the fat diet. This observation may afford the clue to the cause of the low output of uric acid on a fat diet when the carbohydrate supply is defective. The acidosis which results probably brings about a reduction in the alkalinity of the body fluids and either checks the output of uric acid or increases its chances of disintegration.

Macleod and Haskins in their experiments found that the increased output of purine continued for some days after the cessation of the administration of the alkali, but this increase was not observed in the present experiments.

Purine bases. There is no very definite evidence of any influence of the alkali on the output of the bases.

Experiment IV.

In this experiment the influence of glycerol on the output of uric acid and purines was to be tested on two diets, in one of which fat predominated and in the other carbohydrate. Unfortunately only the carbohydrate-rich

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diet can be reported as the subject became ill during the course of the fat-rich diet.

Milk	500 cc.	Protein	79 g.
Rice	500 g.	Carbohydrate	425 „
Water biscuit	220 „	Fat	32 „
Boiled egg (white & yolk)	106 „	Calorie intake	2364 cal.
NaCl	5 „		

TABLE IV.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Aceto-acetic acid g. reckoned as acetone	Test for aceto-acetic acid	Remarks
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines			
I	Acidic	9·16	0·64	0·104	0·014	0·118	6·99	1·14	0·15	1·29	0	–	
II	„	9·30	0·66	0·108	0·018	0·126	7·11	1·16	0·19	1·35	0	–	
III	„	9·07	0·61	0·099	0·021	0·120	6·73	1·09	0·23	1·32	0	–	
IV	„	8·85	0·70	0·097	0·022	0·119	7·91	1·10	0·25	1·34	0	–	30 g. glycerol taken
V	„	8·43	0·77	0·126	0·013	0·139	9·13	1·49	0·15	1·64	0	–	50 g. „ „
VI	„	8·65	0·89	0·122	0·021	0·143	10·29	1·41	0·24	1·65	0	–	50 g. „ „
VII	„	8·60	0·72	0·127	0·018	0·145	8·37	1·48	0·21	1·69	0	–	
VIII	„	9·20	0·64	0·101	0·021	0·122	6·96	1·10	0·23	1·33	0	–	

30 g. of glycerol were taken on the fourth day and 50 g. on the fifth and sixth days.

There seems to be a slight reduction in the total nitrogen output as the result of the taking of glycerol, whereas in the case of the ammonia output, for some unknown reason, it seems to be quite definitely increased. The average output for the three pre-days is 0·64 g. N and for the three glycerol days 0·75 g. The effect is even more clearly seen when the percentage figures are considered where a rise from 6·94 % to 9·11 % occurs.

Uric acid. Horbaczewski and Kanera [1892] found that the output of uric acid was increased by the ingestion of glycerol, and this observation was confirmed in the present experiment. The average output for the three pre-days was 0·104 g. N and, although the output fell to 0·097 g. on the first glycerol day, the average for the three days was 0·115 g. or for the second and third days 0·124 g. The increase is also clearly seen in the percentage figures. It is possible that this increase is due to a synthesis of uric acid, the glycerol in the course of its catabolism giving rise to a certain amount of lactic acid.

Purine bases. The output of bases is apparently uninfluenced by the ingestion of glycerol.

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PART II.

It is a well-known fact that the amount of uric acid in the urine of the dog is minimal, its place being taken by allantoin. This substance was found for the first time in normal dog's urine by Meissner [1868]. Salkowski [1876] was the first worker to show that the output of allantoin in the dog was increased by the administration of uric acid. Cohn [1898] obtained a similar result after feeding with thymus. Minkowski [1898] and Mendel and Brown [1900] have also confirmed this observation. Wiechowski [1907] obtained further confirmation by perfusing isolated organs with blood containing uric acid and obtaining allantoin. He stated [1908] that allantoin was to be considered as a normal end product of purine metabolism in the dog, cat, rabbit and monkey. Underhill and Kleiner [1908] showed that the allantoin present could be derived from endogenous purines as well as from exogenous. As regards the constancy of the daily excretion, this has been demonstrated by Wiechowski and by Hunter and Givens [1910].

As it was of interest to find whether carbohydrate had any influence on the amount of allantoin excreted, a series of experiments was carried out on a large Airedale bitch. The animal was kept in a suitable metabolism cage. She was catheterised at 10 A.M. each day and this urine was added to that collected in the cage. The methods employed were: total nitrogen, Kjeldahl; urea, Plimmer and Skelton's modification of urease method [1914]; ammonia, Folin; total purines, Camerer-Arnstein; allantoin by the method suggested by Plimmer and Skelton [1914].

The animal was fed on a purine- and allantoin-free diet. (Ackroyd [1911] states that milk contains a trace of allantoin but the amount is so small that the diet employed may be considered allantoin-free.)

Experiment V.

Diets employed:

A		*B*		*C*	
Oatmeal	70 g.	Oatmeal	30 g.	Oatmeal ...	30 g.
Margarine ...	80 „	Tapioca	100 „	Dry skimmed milk	30 „
Dry skimmed milk	30 „	Cane sugar ...	80 „	Caseinogen ...	7 „
NaCl	2 „	Dry skimmed milk	50 „	Margarine ...	100 „
		Margarine ...	10 „	NaCl	2 „
		NaCl	2 „		
Protein	22 g.	Protein	23 g.	Protein ...	23 g.
Carbohydrate ...	60 „	Carbohydrate ...	206 „	Carbohydrate ...	34 „
Fat	73 „	Fat	12 „	Fat	86 „
Calorie intake ...	1015 cal.	Calorie intake ...	1051 cal.	Calorie intake ...	1034 cal.

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In the three periods of this diet the protein content was kept approximately constant whilst the carbohydrate-fat content was varied. The animal consumed its food in periods *A* and *B* with avidity but in period *C* (fat-rich, carbohydrate-poor diet) after the sixth day it always left some in the dish; it also developed mild diarrhoea. Accordingly in Table V*C* only the analyses for the first five days, which were free from objection, are recorded.

TABLE V *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·72	1·72	0·15	0·018	0·26	63·24	5·51	0·66	9·56
II	,,	2·32	1·65	0·19	0·020	0·27	71·12	8·19	0·86	7·33
III	,,	2·87	1·95	0·32	0·021	0·22	67·94	11·15	0·73	7·67
IV	,,	2·94	1·93	0·27	0·019	0·24	65·65	9·18	0·65	8·16
V	,,	2·87	1·78	0·29	0·016	0·35	62·02	10·10	0·56	12·20
VI	,,	2·78	1·71	0·31	0·010	0·31	61·51	10·43	0·36	11·15
VII	,,	2·62	1·48	0·18	0·012	0·23	56·49	11·83	0·46	8·78
VIII	,,	2·62	1·71	0·24	0·017	0·31	65·27	6·87	0·65	11·83
IX	,,	2·67	1·66	0·24	0·016	0·32	62·17	8·99	0·60	11·99
X	,,	2·73	1·70	0·31	0·013	0·21	62·27	11·36	0·48	7·69
Average		(2·71)	(1·73)	(0·26)	(0·016)	(0·26)	(64·77)	(9·36)	(0·60)	(9·64)

TABLE V *B*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·21	1·32	0·19	0·017	0·39	59·72	8·60	0·77	17·65
II	,,	2·38	1·27	0·33	0·027	0·40	53·36	13·87	1·22	16·81
III	,,	1·95	1·33	0·19	0·018	0·38	68·21	9·74	0·92	19·49
IV	,,	2·33	1·31	0·38	0·019	0·30	56·22	16·31	0·82	12·88
V	,,	2·38	1·47	0·26	0·022	0·37	61·76	10·92	0·92	15·55
VI	,,	1·66	1·28	0·38	0·023	0·33	77·11	22·82	1·39	19·88
VII	,,	2·07	1·24	0·22	0·018	0·29	59·90	10·63	0·87	14·01
VIII	,,	2·20	1·26	0·28	0·024	0·41	57·27	12·73	1·09	18·64
IX	,,	2·12	1·24	0·25	0·023	0·33	58·49	11·79	1·08	15·57
X	,,	2·20	1·40	0·22	0·022	0·34	63·64	10·00	1·00	15·45
Average		(2·15)	(1·31)	(0·27)	(0·021)	(0·35)	(61·77)	(12·75)	(1·01)	(16·59)

TABLE V *C*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·57	1·70	0·27	0·016	0·16	66·15	10·51	0·62	6·23
II	,,	2·73	1·89	0·19	0·017	0·17	69·23	6·96	0·62	6·23
III	,,	2·30	1·55	0·23	0·016	0·24	67·39	10·00	0·70	10·43
IV	,,	2·64	1·85	0·21	0·019	0·22	70·76	7·95	0·72	8·33
V	,,	2·79	1·96	0·19	0·018	0·20	70·25	6·81	0·65	7·17
Average		(2·61)	(1·79)	(0·22)	(0·017)	(0·20)	(68·76)	(8·45)	(0·66)	(7·68)

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Reaction of urine. Throughout all three periods the reaction remained amphoteric.

Total nitrogen. Here again the protein-sparing action of the carbohydrate is clearly demonstrated, the output is lowest in V *B*. The same result is found in the case of the urea, the lowest amount excreted being on the carbohydrate-rich diet. The effect is seen very clearly in the percentage figures.

Ammonia. Throughout all three periods the output of ammonia tended to be rather irregular. But one point is quite clear, namely the ammonia output on the fat-rich diet gives no evidence of any acidosis. It is of course well known that it is difficult to induce an acidosis in a dog.

Purines. There is certainly a slightly increased output of basal purine in the carbohydrate-rich diet (V *B*) but the increase is not very great.

Allantoin. This substance forms the greatest part of the products of purine metabolism excreted by the dog, and as will be seen from the Tables V *A*, *B* and *C* the amount excreted is apparently dependent on the amount of carbohydrate present in the diet. The output is highest in the case of the carbohydrate-rich diet and is lowest in the carbohydrate-poor, being 0·35 g. nitrogen in V *B*, 0·26 g. in V *A*, and 0·20 g. in V *C*. This variation is seen even more distinctly when the percentage figures are examined: 16·59 %, 9·64 % and 7·68 % respectively. In other words it would seem that the allantoin output in dog's urine behaves just as the uric acid in the human urine. In the case of the dog the question of the part played by acidosis need not be considered, the increased output of allantoin being ascribed solely to the variation in the carbohydrate intake.

Experiment VI.

This experiment was carried out for the purpose of confirming the previous one.

Diets employed:

A		*B*		*C*	
Oatmeal	30 g.	Oatmeal	40 g.	Oatmeal	70 g.
Dry skimmed milk	30 „	Dry skimmed milk	30 „	Dry skimmed milk	30 „
Caseinogen ...	9 „	Tapioca	98 „	Caseinogen ...	3 „
Margarine ...	99 „	Margarine ...	50 „	Margarine ...	81 „
NaCl	2 „	Caseinogen ...	7 „	NaCl	2 „
		NaCl	2 „		
Protein	25 g.	Protein	25 g.	Protein	25 g.
Carbohydrate ...	34 „	Carbohydrate ...	125 „	Carbohydrate ...	60 „
Fat	85 „	Fat	45 „	Fat	74 „
Calorie intake ...	1032 cal.	Calorie intake ...	1034 cal.	Calorie intake ...	1037 cal.

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TABLE VI *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	3·04	2·35	0·36	0·026	0·10	77·30	11·84	0·86	3·29
II	,,	3·04	2·02	0·52	0·022	0·16	68·42	17·11	0·72	5·26
III	,,	2·86	1·99	0·36	0·019	0·12	69·58	12·59	0·66	4·20
IV	,,	2·92	1·99	0·38	0·021	0·18	68·15	13·01	0·72	6·16
V	,,	2·76	1·85	0·39	0·018	0·14	67·03	14·13	0·65	5·07
Average		(2·92)	(2·04)	(0·40)	(0·021)	(0·14)	(70·10)	(13·74)	(0·72)	(4·80)

TABLE VI *B*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·55	1·39	0·33	0·016	0·34	54·51	12·94	0·63	13·33
II	,,	2·24	1·44	0·30	0·021	0·30	64·29	13·39	0·94	13·39
III	,,	2·21	1·54	0·27	0·013	0·27	69·68	12·22	0·59	12·22
IV	,,	2·44	1·78	0·34	0·012	0·32	72·95	13·93	0·49	13·11
V	,,	2·63	1·53	0·30	0·012	0·29	58·17	11·41	0·46	11·03
Average		(2·41)	(1·54)	(0·31)	(0·015)	(0·30)	(63·92)	(12·78)	(0·62)	(12·62)

TABLE VI *C*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·70	2·03	0·30	0·015	0·22	75·19	11·11	0·56	8·15
II	,,	2·54	1·93	0·20	0·019	0·18	75·98	7·87	0·75	7·09
III	,,	2·49	1·42	0·30	0·015	0·23	57·03	12·05	0·60	9·24
IV	,,	2·60	1·62	0·23	0·015	0·22	62·31	8·85	0·58	8·46
V	,,	2·98	2·24	0·29	0·016	0·26	75·17	9·73	0·54	8·72
Average		(2·66)	(1·85)	(0·26)	(0·016)	(0·22)	(69·14)	(9·23)	(0·61)	(8·33)

The outputs of total nitrogen and urea agree with the previous results, but for some reason the output of ammonia on the carbohydrate-rich diet VI *B* is higher than on VI *C*. In the case of the purine bases the output is highest on the fat-rich diet and no conclusion regarding the influence of food on the output of bases can be reached.

Allantoin. The result here is similar to that in the previous experiment, i.e. the output is greatest with the diet containing most carbohydrate.

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Experiment VII.

It has been clearly shown by Minkowski [1886], Wiener [1901], Kowalewski and Salaskin [1901] and others that in the avian organism at least it can be readily demonstrated that uric acid is formed by the condensation of lactic acid with urea, and Wiener has maintained that the same process can take place in the mammalian organism. All previous experiments so far as I am aware have been based on the assumption that uric acid was an end product, and in this experiment attention was directed to the influence of the ingestion of lactic acid on the output of allantoin, the normal end product of purine metabolism in the dog.

A fat-rich carbohydrate-poor diet was selected, as the normal allantoin output would be low and thus the influence of the ingested lactic acid could be more readily detected.

Diet:

Oatmeal	30 g.	Protein	25 g.
Dry skimmed milk	30 „	Carbohydrate	34 „
Caseinogen	9 „	Fat	84 „
Margarine	100 „	Calorie intake	1032 cal.
NaCl	2 „		

TABLE VII.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Remarks
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin	
I	Amphoteric	2·84	1·90	0·39	0·022	0·18	66·90	13·73	0·77	6·34	
II	„	3·05	1·73	0·31	0·022	0·23	56·72	10·16	0·72	7·54	
III	„	3·31	2·23	0·25	0·021	0·20	67·37	7·55	0·63	6·04	
Average		(3·07)	(1·95)	(0·32)	(0·022)	(0·20)	(63·66)	(10·48)	(0·70)	(6·64)	
IV	„	2·73	1·92	0·27	0·019	0·30	70·33	9·89	0·70	10·99	10 g. sod. lactate
V	„	2·80	2·09	0·25	0·020	0·25	74·64	8·93	0·71	8·93	„ „
VI	„	2·67	1·83	0·25	0·021	0·31	68·54	9·36	0·79	11·61	„ „
Average		(2·73)	(1·95)	(0·26)	(0·020)	(0·28)	(71·17)	(9·39)	(0·73)	(10·51)	
VII	„	2·60	1·77	0·30	0·020	0·25	68·08	11·54	0·77	9·62	
VIII	„	2·48	1·74	0·29	0·016	0·15	70·16	11·69	0·65	6·05	
IX	„	2·42	1·76	0·32	0·016	0·13	72·73	13·22	0·66	5·37	
Average		(2·50)	(1·76)	(0·30)	(0·017)	(0·18)	(70·32)	(12·15)	(0·69)	(7·03)	
X	„	2·46	1·58	0·28	0·016	0·36	64·23	11·38	0·61	14·63	20 g. sod. lactate
XI	„	2·55	1·72	0·32	0·018	0·33	67·45	12·55	0·71	12·94	„ „
XII	„	2·79	2·05	0·30	0·017	0·27	73·48	10·75	0·61	9·68	„ „
Average		(2·60)	(1·78)	(0·30)	(0·017)	(0·32)	(68·39)	(11·56)	(0·64)	(12·42)	
XIII	„	2·73	1·76	0·28	0·017	0·18	64·47	10·26	0·62	6·59	
XIV	„	2·65	1·63	0·35	0·017	0·18	61·51	13·21	0·64	6·79	
Average		(2·69)	(1·70)	(0·32)	(0·017)	(0·18)	(62·99)	(11·74)	(0·63)	(6·69)	

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On the mornings of the fourth, fifth and sixth days 10 g. of sodium lactate and on the tenth, eleventh and twelfth days 20 g. of sodium lactate were given.

The ingestion of the sodium lactate is apparently without influence on the output of total nitrogen, urea, ammonia and purine bases.

Allantoin. In the case of this substance the administration of the sodium lactate seems to exert a very direct influence. The average output during the first three normal days was 0·20 g. allantoin nitrogen, this rose to an average output of 0·29 g. after the giving of 10 g. sodium lactate; on the three following normal days the output declined to an average of 0·18 g. and rose again to 0·32 g. on giving 20 g. of sodium lactate. The output on the two following normal days returned to its old level of 0·18 g. The result is also clearly seen when the figures of the percentage of total nitrogen are considered. Although, then, the actual amounts of allantoin excreted are not perhaps very large they show a definite increase following the ingestion of sodium lactate and it would therefore seem probable that an actual synthesis of uric acid had taken place.

Conclusions.

1. The protein-sparing action of carbohydrate as compared with fat is again clearly demonstrated in this series of experiments. Glycerol would seem also to act as a protein sparer.

2. In the case of man there is some evidence of the synthetic formation of uric acid when the diet is rich in carbohydrate. In the dog the same result is obtained in the case of allantoin.

3. The output of endogenous uric acid in the human urine is increased after the ingestion of glycerol.

4. There is a distinct rise in the output of allantoin in the dog's urine after the administration of sodium lactate.

5. The output of endogenous uric acid is increased by the giving of sodium bicarbonate.

6. In several experiments it was found that a decrease in the output of purine bases was associated with an increase in the excretion of uric acid.

7. In fat-rich diets which are carbohydrate-poor the output of uric acid is markedly diminished.

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XLII. THE INFLUENCE OF CARBOHYDRATE AND FAT ON PROTEIN METABOLISM WITH SPECIAL REFERENCE TO THE OUTPUT OF SULPHUR.

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(*Received October 20th, 1915.*)

In view of the fact that carbohydrate brings about a very definite reduction in the output of nitrogen in the urine and that fat tends to raise this output, when carbohydrate is present in small amount, it was of interest to find how the sulphur output would behave under similar conditions. The N : S ratio has now been repeatedly investigated, with special reference to the rate at which these two substances are excreted [Siven, 1901; Sherman and Hawk, 1901; von Wendt, 1905; Ehrström, 1906; Falta, 1906; Hämäläinen and Helme, 1907; Wolf, 1912; and Cathcart and Green, 1913]. These observers however do not all agree in their conclusions, some maintain that the nitrogen and sulphur are excreted at a parallel rate, whilst others hold that the sulphur appears before the nitrogen. Whatever the rate, the general conclusion of von Wendt is well founded, viz. that the only true picture of the total protein exchange in the body is obtained when the nitrogen and sulphur excretions are considered together.

As regards the partition of the sulphur present in the urine, Folin's [1905] conclusions may be accepted. He found that when the total sulphur excretion falls, the percentage of sulphur present as inorganic sulphates diminishes—these bear almost the same relationship to total sulphur as urea does to total nitrogen, i.e. they represent the products which are chiefly involved in quantitative changes of the total sulphur. The ethereal sulphates and neutral sulphur would seem to have an origin analogous to that of purines and creatinine. Folin found that the ethereal sulphate output becomes

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more prominent when the food contains little or no protein and that the neutral sulphur represents products "which in the main are independent of the total amount of sulphur eliminated or of protein katabolised."

The method employed in the present experiment was that of superimposition, i.e. on a particular day there was added to a standard diet the special foodstuff to be tested, the content of which in nitrogen and sulphur was known. In different cases the diet was rich in fat and correspondingly poor in carbohydrate, or fat-poor and carbohydrate-rich. As the subject of the experiment a healthy bitch weighing approximately 13 kilo. was employed. The animal was kept in a metabolic cage in the usual way. She was catheterised each morning at ten and the urine added to that collected in the receiver of the cage. The urine, after the quantity, reaction, and specific gravity had been determined, was always made up to 800 cc. with water.

The diets employed were as follows:

Carbohydrate-rich		Fat-rich	
Oatmeal	70 g.	Oatmeal	70 g.
Tapioca	100 g.	Dry skimmed milk ...	30 g.
Cane sugar	40 g.	Margarine	90 g.
Dry skimmed milk ...	30 g.	Water	500 cc.
Margarine	20 g.		
Water	500 cc.		

The animal was allowed to have drinking water *ad libitum.*

The methods of analysis employed were: total nitrogen, Kjeldahl; total sulphur, Benedict; inorganic and ethereal sulphates, Folin. The faeces were not examined.

The standard diet was continued until the output of total nitrogen and total sulphur became fairly regular. Then an amount of the protein to be tested, which contained approximately 6 g. of nitrogen, was added to the standard diet at the usual feeding time. The standard diet was then continued until the output of total nitrogen and of total sulphur was again regular. The extra amounts of nitrogen and sulphur excreted were determined by the subtraction of the average amount of nitrogen and sulphur excreted on the three days immediately preceding the superimposition from that excreted after the addition.

Experiment I. Addition of Caseinogen.

The amount of caseinogen was 50 g., containing 6·21 g. total nitrogen, 0·482 g. of total sulphur (0·179 g. in the form of oxidised sulphur), thus giving an S : N ratio of 1 : 12·8. The result of this superimposition in the case of the two diets is seen in the following tables.

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TABLE I. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g.				Per cent. of T. sulphur			T.S. : T.N.	Remarks
					T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	Inorganic sulphate	Ethereal sulphate	Neutral sulphur		
v	470	acid	1010	2·43	0·166	0·079	0·012	0·075	47·6	7·2	45·2	1 : 14·6	
vi	520	amph.	1009	2·41	0·166	0·071	0·013	0·082	42·8	7·8	49·4	1 : 14·5	
vii	540	acid	1008	2·44	0·168	0·074	0·011	0·083	44·0	6·5	49·4	1 : 14·5	
viii	560	,,	1011	4·00	0·241	0·150	0·013	0·078	62·2	5·5	31·3	1 : 16·6	50 g. caseinogen
ix	640	,,	1006	2·54	0·187	0·092	0·013	0·083	48·9	6·8	44·3	1 : 13·6	
x	580	,,	1009	2·21	0·163	0·075	0·009	0·080	45·6	5·4	49·0	1 : 13·8	
xi	600	,,	1008	2·16	0·160	0·072	0·009	0·079	44·8	5·9	49·3	1 : 13·5	
xii	630	amph.	1006	2·18	0·146	0·064	0·011	0·071	43·6	7·5	48·8	1 : 15·0	
xiii	630	acid	1006	2·16	0·150	0·066	0·011	0·072	44·4	7·6	48·0	1 : 14·5	

TABLE II. (*Fat diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g.				Per cent. of T. sulphur			T.S. : T.N.	Remarks
					T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	Inorganic sulphate	Ethereal sulphate	Neutral sulphur		
iv	440	acid	1013	2·42	0·151	0·064	0·015	0·072	42·6	9·6	47·9	1 : 16·0	
v	640	amph.	1008	2·54	0·160	0·072	0·016	0·072	44·7	10·1	45·2	1 : 15·9	
vi	550	acid	1010	2·59	0·145	0·063	0·013	0·068	43·6	9·1	47·3	1 : 17·9	
vii	1040	,,	1007	5·55	0·341	0·233	0·018	0·089	68·5	5·3	26·2	1 : 16·3	50 g. caseinogen
viii	850	,,	1005	2·99	0·178	0·090	0·015	0·073	50·6	8·4	41·0	1 : 16·8	
ix	570	,,	1012	2·64	0·168	0·080	0·014	0·073	47·8	8·4	43·8	1 : 15·8	
x	530	,,	1012	2·22	0·129	0·054	0·012	0·063	41·7	9·6	48·7	1 : 17·2	
xi	480	,,	1011	2·42	0·143	0·061	0·015	0·066	43·1	10·5	46·5	1 : 17·0	

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The extra-elimination of the nitrogen and the sulphur is shown in Tables I *A* and II *A*.

TABLE I *A*. (*Carbohydrate diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	1·57	0·074	0·075	0·001	0·002	1 : 21·1
First day	0·11	0·020	0·017	0·001	0·003	1 : 5·5
Total	1·68	0·094	0·092	0·002	0·005	1 : 17·9

TABLE II *A*. (*Fat diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	3·03	0·189	0·167	0·003	0·018	1 : 16·0
First day	0·47	0·026	0·024	—	0·002	1 : 18·8
Second day	0·12	0·016	0·014	0·001	0·002	1 : 7·5
Total	3·62	0·231	0·205	0·004	0·022	1 : 15·6

There is a definite rise in the output of total nitrogen and total sulphur after the superimposition, most marked on the day of feeding on both diets, and to a slight extent on the first day after with the carbohydrate diet, and on the first and second on the fat diet. This excess forms only 27 % of the nitrogen ingested with the carbohydrate diet: thus there is well-marked retention. But the retention is even more definite when the sulphur moiety is considered, as in the two days there is an excretion of only 0·094 g. total sulphur, i.e. 19·5 % of the sulphur ingested. When the fat diet is considered the results are different, as in the case of the nitrogen 58 % of that ingested is excreted and 48 % of the sulphur. In each instance the bulk of the sulphur excreted is in the form of inorganic sulphates.

As regards the other question, as to whether the carbohydrate-rich or fat-rich diet affects the distribution of the sulphur, there is no doubt about the fact that in this experiment when the outputs on the two diets are compared, although the total nitrogen output is slightly lower on the carbohydrate diet, the output of total sulphur is somewhat higher. As regards the partition of the sulphur it is of interest to note that on both diets about half of the sulphur excreted is in the form of neutral sulphur. Taking the mean of the three pre-days, it is found on the carbohydrate diet that 44·8 % is in the form of inorganic sulphates, 7·2 % as ethereal sulphates and 48·0 % as neutral sulphur. On the fat diet 43·6 % is present as inorganic sulphates, 9·6 % as ethereal sulphates, and 46·8 % as neutral sulphur. There is a slight

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increase both in absolute and percentage amount in the output of ethereal sulphates on the fat diet.

Experiment II. Egg albumin.

50 g. of finely ground egg albumin containing 6·14 g. of nitrogen and 0·671 g. of total sulphur (with 0·041 g. in the oxidised form) were superimposed on the two standard diets as in the previous experiment. Unfortunately the fat diet could not be carried on until the old level was reached as the dog after the twelfth day refused to eat the food. The results are found in Tables III and IV.

The extra-elimination of the nitrogen and the sulphur is shown in Tables III *A* and IV *A*.

In the present experiment there is quite a marked difference between the output of the extra nitrogen and sulphur on the carbohydrate and fat diets. It required in the case of the carbohydrate-rich diet three days for the excretion of the excess nitrogen, 41·5 % of that ingested being turned out in this time, whereas the excretion of the sulphur continued quite definitely for seven days, 49·5 % of that ingested being excreted. With the fat diet this disparity of excretion was not found although the excretion of the nitrogen was continued over five days, with the result that 56·7 % of that ingested was turned out. In the case of the sulphur this also continued for five days, 47·4 % of that ingested being excreted.

Here then again, just as in the case of the caseinogen, the conditions for the retention of nitrogen on a carbohydrate diet are better than in the case of a fat diet, a difference of 31 % with the caseinogen and over 15 % with the egg albumin.

As in the previous experiment, the great bulk of the sulphur excreted is in the form of inorganic sulphates.

In this experiment not only is the total nitrogen output on the carbohydrate diet quite definitely lower but so is also the total sulphur output. The ratio of the output of sulphur to nitrogen in spite of these differences is practically identical in both experiments. It is therefore simply a clear case of complete reduction of the total protein catabolism.

When the form in which the sulphur is excreted is considered it will be noted that although on both diets the output is practically distributed between the inorganic sulphates and the neutral sulphur, in the case of the carbohydrate diet the greater part is in the form of neutral sulphur, whereas on the fat diet the greater part is present as inorganic sulphates.

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TABLE III. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g. T.S.	S. in g. Inorganic sulphate	S. in g. Ethereal sulphate	S. in g. Neutral sulphur	Per cent. of T. sulphur Inorganic sulphate	Per cent. of T. sulphur Ethereal sulphate	Per cent. of T. sulphur Neutral sulphur	T.S. : T.N.	Remarks
vii	570	amph.	1009	2·35	0·126	0·051	0·010	0·065	40·5	7·9	51·6	1 : 18·6	
viii	610	„	1008	2·33	0·133	0·056	0·012	0·065	42·1	9·0	48·9	1 : 17·5	
ix	500	„	1009	2·33	0·130	0·057	0·010	0·063	43·8	7·7	48·5	1 : 17·9	
x	550	„	1014	4·37	0·273	0·188	0·018	0·067	68·9	6·6	24·5	1 : 16·0	50 g. egg albumin
xi	560	acid	1010	2·74	0·214	0·123	0·018	0·073	57·5	8·4	34·1	1 : 12·8	
xii	670	amph.	1008	2·45	0·171	0·088	0·015	0·068	51·5	8·8	39·8	1 : 14·3	
xiii	530	„	1011	2·24	0·150	0·067	0·012	0·071	44·7	8·0	47·3	1 : 14·9	
xiv	590	„	1007	2·32	0·150	0·069	0·014	0·067	46·0	9·3	44·7	1 : 15·5	
xv	590	„	1008	2·27	0·144	0·066	0·012	0·066	45·8	8·3	45·8	1 : 15·8	
xvi	610	acid	1011	2·37	0·140	0·064	0·013	0·063	45·7	9·3	45·0	1 : 17·1	
xvii	710	amph.	1006	2·37	0·130	0·055	0·013	0·062	42·3	10·0	47·7	1 : 18·2	

TABLE IV. (*Fat diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g. T.S.	S. in g. Inorganic sulphate	S. in g. Ethereal sulphate	S. in g. Neutral sulphur	Per cent. of T. sulphur Inorganic sulphate	Per cent. of T. sulphur Ethereal sulphate	Per cent. of T. sulphur Neutral sulphur	T.S. : T.N.	Remarks
v	490	alk.	1014	2·67	0·163	0·088	0·011	0·064	54·0	6·7	39·3	1 : 16·4	
vi	680	„	1009	2·52	0·145	0·069	0·011	0·065	47·6	7·6	44·8	1 : 17·4	
vii	740	„	1007	2·60	0·136	0·063	0·010	0·063	46·3	7·4	46·3	1 : 19·1	
viii	1050	amph.	1007	4·20	0·275	0·174	0·014	0·087	63·3	5·1	31·6	1 : 15·3	50 g. egg albumin
ix	690	alk.	1010	3·08	0·262	0·161	0·015	0·086	61·5	5·7	32·8	1 : 11·8	
x	960	amph.	1006	2·90	0·181	0·094	0·011	0·076	51·9	6·1	42·0	1 : 16·0	
xi	1100	„	1007	3·25	0·176	0·093	0·010	0·073	52·8	5·7	41·5	1 : 18·5	
xii	1000	„	1005	3·05	0·164	0·080	0·012	0·072	48·8	4·3	43·9	1 : 18·6	

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TABLE III *A*. (*Carbohydrate diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	2·03	0·143	0·133	0·007	0·003	1 : 14·2
First „	0·40	0·084	0·068	0·007	0·009	1 : 4·8
Second „	0·11	0·041	0·033	0·004	0·004	1 : 2·7
Third „	—	0·020	0·012	0·001	0·007	—
Fourth „	—	0·020	0·014	0·003	0·003	—
Fifth „	—	0·014	0·011	0·001	0·002	—
Sixth „	—	0·010	0·009	0·002	−0·001	—
Total	2·54	0·332	0·280	0·025	0·027	1 : 7·6

TABLE IV *A*. (*Fat diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	1·60	0·127	0·101	0·003	0·023	1 : 12·6
First „	0·48	0·114	0·088	0·004	0·022	1 : 4·2
Second „	0·30	0·033	0·021	—	0·012	1 : 9·1
Third „	0·65	0·028	0·020	−0·001	0·009	1 : 23·2
Fourth „	0·45	0·016	0·007	0·001	0·008	1 : 28·1
Total	3·48	0·318	0·237	0·007	0·074	1 : 10·9

Experiment III. Gelatin.

As in both of the previous experiments the extra output of total nitrogen was higher on the fat than on the carbohydrate diet, i.e. more perfect retention having taken place on the latter diet, it was of some interest to see whether when an "imperfect" protein like gelatin was employed a similar disparity would occur. In this experiment 40 g. of gelatin containing 5·86 g. of nitrogen and 0·541 g. of sulphur (0·109 in an oxidised form) were dissolved in water and superimposed on the standard diets (Tables V and VI).

The extra-elimination of the nitrogen and the sulphur is shown in Tables V *A* and VI *A*.

As in the other experiments there is again a greater output of the superimposed nitrogen in the case of the fat diet; on the carbohydrate diet 78·3 % of the gelatin nitrogen ingested reappeared in the urine in the course of three days, and on the fat diet within the same period 88·7 % reappeared. In the case of the sulphur the result was very similar, on the carbohydrate diet 24·7 % and on the fat diet 30·3 % was excreted, in both instances the extra output, mainly in the form of inorganic sulphates, was confined to the fed-day.

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TABLE V. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g.				Per cent. of T. sulphur			T.S. : T.N.	Remarks
					T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	Inorganic sulphate	Ethereal sulphate	Neutral sulphur		
iv	930	amph.	1008	2·03	0·141	0·056	0·015	0·070	39·7	10·6	49·6	1 : 14·4	
v	820	,,	1009	2·04	0·137	0·051	0·017	0·069	37·2	12·4	50·4	1 : 14·9	
vi	1050	,,	1007	2·16	0·141	0·058	0·016	0·067	41·1	11·3	47·5	1 : 15·3	
vii	1000	,,	1009	5·94	0·274	0·170	0·016	0·088	62·0	5·9	32·1	1 : 21·7	40 g. gelatin
viii	670	,,	1010	2·60	0·118	0·039	0·013	0·066	33·1	11·0	55·9	1 : 22·0	
ix	950	,,	1007	2·29	0·126	0·043	0·016	0·067	34·1	12·7	53·2	1 : 18·2	
x	730	,,	1008	2·08	0·144	0·060	0·017	0·067	41·7	11·8	46·5	1 : 14·4	
xi	790	,,	1008	2·18	0·153	0·071	0·015	0·067	46·4	9·8	43·8	1 : 14·3	

TABLE VI. (*Fat diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g.				Per cent. of T. sulphur			T.S. : T.N.	Remarks
					T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	Inorganic sulphate	Ethereal sulphate	Neutral sulphur		
ii	800	amph.	1009	2·32	0·145	0·062	0·013	0·070	42·8	9·0	48·3	1 : 16·0	
iii	700	,,	1010	2·29	0·157	0·070	0·016	0·071	44·6	10·2	45·2	1 : 14·5	
iv	1100	,,	1005	2·54	0·162	0·071	0·016	0·075	43·8	9·9	46·3	1 : 15·7	
v	1360	acid	1010	6·68	0·319	0·213	0·018	0·088	66·8	5·6	27·6	1 : 20·9	40 g. gelatin
vi	850	amph.	1007	3·06	0·140	0·057	0·015	0·068	40·7	10·7	48·6	1 : 21·9	
vii	1000	,,	1006	2·60	0·149	0·063	0·015	0·071	42·3	10·1	47·7	1 : 17·4	
viii	1000	acid	1006	2·23	0·135	0·051	0·014	0·070	37·8	10·4	51·9	1 : 16·5	

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TABLE V *A*. (*Carbohydrate diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	3·86	0·134	0·115	—	0·019	1 : 28·8
First „	0·52	—	—	—	—	—
Second „	0·21	—	—	—	—	—
Total	4·59	0·134	0·115	—	0·019	1 : 34·3

TABLE VI *A*. (*Fat diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	4·30	0·164	0·145	0·003	0·016	1 : 26·2
First „	0·68	—	—	—	—	—
Second „	0·22	—	—	—	—	—
Total	5·20	0·164	0·145	0·003	0·016	1 : 31·7

It is of interest to note that the output of extra total nitrogen after the gelatin feeding is very definitely higher than is the case with the more "perfect" proteins caseinogen and egg albumin. It is difficult to say why the retention of sulphur is more marked than in the other experiments. It is of course possible that the standard diet, on which the animal was mainly living, was poor in sulphur and that there was a real demand for this substance. It must not be forgotten that in all probability the demand for sulphur by an animal like the dog is relatively greater than in the case of man, due to the fact that there is always a very steady loss of sulphur-rich material like hair.

These results certainly do not agree with many previous experiments, which however were carried out on human beings, where it has been practically uniformly found that if the output of sulphur did not actually exceed the total nitrogen, it at the very least ran parallel with it.

As regards the output of nitrogen and sulphur on the standard diet in this experiment, just as in Exp. II on the carbohydrate diet, the output of both nitrogen and sulphur is lower than on the fat diet. With respect to the partition of the sulphur on both diets, neutral sulphur is the most predominant constituent, although in both ethereal sulphates are excreted in greater amount than in either of the other two experiments.

These experiments not only bear out the statement first put forward by Falta and since confirmed by others that the rate of the excretion of the catabolic products of protein depends on the nature of the protein ingested,

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but they allow of the further amplification that the rate at which these products are excreted depends not only on the nature of the protein but also on the nature of the diet on which the protein has been superimposed.

These experiments were carried out at the suggestion and under the direction of Dr E. P. Cathcart, to whom I offer my thanks.

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Summary.

1. In all three experiments the retention of superimposed nitrogen is greater on a carbohydrate than on a fat diet.

2. In two experiments out of the three the same holds good for sulphur.

3. The amount of extra nitrogen and sulphur excreted varies with the protein used.

4. The greater part of the extra sulphur excreted is in the form of inorganic sulphates.

5. The partition of sulphur on the carbohydrate and fat diets is very similar—it is for the most part excreted in the form of inorganic sulphates and neutral sulphur in almost equal amount.

6. There is no evidence that the protein retained after superimposition is poor in sulphur. Indeed with the exception of one experiment sulphur is definitely retained in larger amount than nitrogen.

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XLIII. THE OUTPUT OF CREATINE IN GLYCOSURIA.

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In order to obtain further evidence in support of the view that the appearance of creatine in the urine in animals, where the metabolism of carbohydrate has been interfered with, is actually due to the disturbance of the metabolic activities, and is not merely an error in observation due to the presence of aceto-acetic acid, the present experiments were carried out.

It has already been shown by Cathcart and Orr [1914] that in the case of the administration of phloridzin a creatinuria can exist without a concomitant acidosis. As the "error" in phloridzin glycosuria is generally considered to be renal, it was of some interest to examine the urine in other forms of glycosuria in which the cause is less obvious. The forms of glycosuria selected were (1) that following the injection of adrenaline, and (2) post-anaesthetic glycosuria.

PART I.

Adrenaline Glycosuria.

Since the discovery by Blum [1901] that the injection of adrenaline caused glycosuria, many confirmatory experiments have been carried out, but there is not yet perfect agreement as regards the mode of action of this substance. Noël Paton [1903] found a break-down of protein and a rise in the output of ammonia after the subcutaneous injection of adrenaline and concluded that the protein metabolism behaved as in ordinary diabetes. Underhill and Clossen [1907] were unable either to detect any increase in the catabolism

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of protein or to find any alteration in the distribution of the nitrogenous constituents of the urine in adrenaline glycosuria. They believed that the action of the adrenaline was to stimulate the sugar-storing organs through the sympathetic nerves and thus prevent the storage of glycogen. The ability of the organism to utilise dextrose subcutaneously administered was not seriously impaired by the injection of adrenaline. From the fact that he was able to produce glycosuria with a dose of adrenaline which would produce a vaso-constriction in the tissues, Ritzmann [1909] concluded that the glycosuria was probably due to some sympathetic stimulation with a mechanism similar to that for vaso-constriction. On the other hand Wilenko [1912] thought that his results pointed directly to the glycosuria being due to some interference with the power of the tissues to oxidise the sugar. He found no rise in the respiratory quotient and maintained that sugar injected into an animal suffering from adrenaline glycosuria appeared in quantitative amount in the urine. Lusk [1913] however did not think that the oxidation was interfered with or that the protein metabolism was increased.

The present experiments were carried out on an Airedale bitch weighing 13·2 kilo. She was kept in an ordinary metabolism cage and fed on a fixed diet, in one experiment carbohydrate-rich and in the other carbohydrate-poor. The animal was catheterised each morning at 10 a.m. and the urine mixed with that collected in the receiver of the cage. It was diluted to a constant volume each day. The methods of analysis employed were: total nitrogen, Kjeldahl; ammonia, creatinine and creatine, Folin; urine treated in the way recommended by Graham and Poulton; urea, urease method; sugar, Benedict; and acetone and aceto-acetic acid, Messinger-Huppert. The adrenaline employed was the 1 : 1000 solution of Parke, Davis and Co.

Exp. I. Carbohydrate-rich diet: 100 g. tapioca, 70 g. oatmeal, 40 g. cane sugar, 30 g. dried skimmed milk, and 20 g. margarine.

As soon as the animal was in nitrogenous equilibrium 7 cc. of the adrenaline solution were injected subcutaneously. As a result of this injection the dog became very restless, and had free salivation but no marked dyspnoea. After the initial excitement passed off she lay down on the floor of her cage and remained quietly there for the rest of the day. She only ate a small part of her food this day. The following day, although she still looked somewhat ill, she ate all her food. On the next day at the site of injection which had become indurated a small centre of necrosis appeared. No albumin appeared in the urine either on the day of injection or any following day.

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TABLE I. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	Urea N. in g.	Urea N. p. c.	NH_3 N in g.	NH_3 N p. c.	Creatinine N. in g.	Creatinine N. p. c.	Creatine N. in g.	Sugar in g.	Acetone and aceto-acetic acid in g.
vi	520	acid	1011	2·85	1·55	54·3	0·143	5·0	0·126	4·4	—	—	—
vii	430	„	1013	2·88	1·52	52·7	0·157	5·5	0·121	4·2	—	—	—
viii	520	„	1011	2·82	1·77	62·8	0·122	4·3	0·129	4·6	—	—	—
ix	1100	„	1011	4·64	2·55	54·9	0·240	5·1	0·159	3·4	0·072	5·85	0·0043
x	1220	„	1008	4·22	2·09	50·0	0·253	6·0	0·177	4·2	0·045	0·61	0·0023
xi	460	„	1018	3·76	1·88	50·0	0·099 (?)	2·6	0·141	3·8	—	—	0·0035
xii	280	„	1018	3·61	1·85	51·2	0·166	4·6	0·114	3·2	0·002	—	—

It will be seen from the table, that as the result of the injection of the adrenaline there is a very definite rise in the output of total nitrogen. This rise is not due to any increase in one particular constituent but to a general increase of all the constituents estimated, as will be noted from the examination of the percentage outputs. As was to be expected, on the day of the injection there was glycosuria and associated with it the appearance of creatine. Although the output of sugar on the day following had fallen to a low level, the output of creatine was still quite marked. That this is a real creatine excretion and not merely apparent, due to the presence of aceto-acetic acid, is clear when the total acetone excreted is considered.

Exp. II. Carbohydrate-poor diet consisting of 70 g. oatmeal, 30 g. dried skimmed milk and 90 g. margarine. The same dose of adrenaline as in the last experiment was given but this time intramuscularly. The results of the injection were practically identical with those in Exp. I. Two days after the injection, in spite of the fact that the injection was into the deeper tissues, a slight necrosis of the skin appeared.

TABLE II. (*Fat diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	Urea N. in g.	Urea N. p. c.	NH_3 N in g.	NH_3 N p. c.	Creatinine N. in g.	Creatinine N. p. c.	Creatine N. in g.	Sugar in g.	Acetone and aceto-acetic acid in g.
v	1050	amph.	1007	2·18	1·48	67·9	0·259	11·9	0·153	7·0	—	—	—
vi	1130	„	1008	2·39	1·30	54·4	0·290	12·1	0·165	6·9	—	—	0·0044
vii	1600	„	1008	3·58	1·88	52·5	0·672	18·7	0·166	4·6	0·101	0·81	0·0070
viii	1300	alk.	1007	2·60	1·21	46·5	0·852	32·8	0·151	5·8	0·023	—	0·0041
ix	1000	„	1007	2·39	1·43	59·8	0·459	19·2	0·150	6·2	—	—	0·0098
x	1000	amph.	1006	2·42	1·57	64·9	0·381	15·7	0·155	6·4	—	—	0·0059

As the result of the injection there is again quite a definite rise in the output of total nitrogen, but in this instance there is a much greater disturbance in the percentage composition. It will be noted that here there is a very marked rise in the output of ammonia and a definite rise in the excretion of total nitrogen and urea. As was to be expected the output of sugar

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is much smaller although there is an increase in the output of creatine as compared with Exp. I. Here again, although there is a slight increase in the output of the total acetone, it is not sufficient to account for the creatine result.

PART II.

Post-anaesthetic Glycosuria.

It is probable that the glycosuria which follows the administration of ether is due to some disturbance of the hepatic metabolic activities. Seelig [1905], although he was unable to prove that glycosuria was a symptom of ether anaesthesia in the human subject, found that in the case of the dog a hyperglycaemia occurred after ether narcosis. As regards the glycosuria he found that it was very transitory, and even did not occur at all if, previous to the administration of the anaesthetic, an intravenous infusion of oxygen was given. He was quite certain that the glycosuria was due to the action of the ether and not to any associated symptoms arising during the narcosis.

King, Moyle and Haupt [1912] came to the conclusion that the glycosuria following ether administration was not due to asphyxia caused by the ether inhalation but was due to the intoxication with ether itself, as intravenous injection of saline saturated with ether also induced glycosuria.

As regards the influence of ether inhalation on the protein metabolism this subject has been fully discussed by Hawk [1908], who, as the result of his own experiments, found that ether anaesthesia was invariably followed by an increase in the excretion of nitrogen.

As in the previous experiments the same two diets were employed, the subject of experiment being an Airedale bitch weighing 11 kilo. On account of the transitory nature of the changes produced by ether anaesthesia, the urine of the day on which the animal was anaesthetised was divided into two parts and separately examined; (*a*) the urine which was excreted and obtained from the bladder by catheter before the anaesthetic was given, and (*b*) the urine collected for the rest of the 24-hour period after the anaesthetic.

Exp. III. Carbohydrate-rich diet. After the animal had reached a state of nitrogen equilibrium it was anaesthetised with ether for 90 minutes, from 2.30 p.m. to 4 p.m.

The animal was given a small dose (2–3 grains) of morphia before the ether was administered. Small doses of morphia have no influence on protein metabolism and have but little influence in causing glycosuria. Following the

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anaesthetic there was slight vomiting but no other symptom. The animal rapidly recovered.

TABLE III. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	N. in g. T.N.	Urea	NH_3	Creatinine	Creatine	Sugar in g.	Acetone and aceto-acetic acid in g.
iv	850	acid	1006	2·25	1·43	0·238	0·148	—	—	—
v	1000	,,	1006	2·21	1·27	0·248	0·147	—	—	0·0029
vi	1030	,,	1005	2·25	1·48	0·260	0·147	—	—	0·0029
vii	220	amph.	1006	0·52	0·31	0·055	0·032	—	—	0·0005
	560	alk.	1014	1·76	1·34	0·045	0·098	0·009	0·45	0·0039
	780			2·28	1·65	0·100	0·130	0·009	0·45	0·0044
viii	800	amph.	1006	2·78	1·97	0·318	0·129	—	—	0·0054
ix	1350	acid	1005	2·29	1·31	0·507	0·138	—	—	—

There is no rise in the output of total nitrogen on the day of narcosis but there is a very slight rise in the output of the following day. There is however a slight rise in the excretion of urea and a very definite fall in the output of ammonia, a slight fall in creatinine, the appearance of a trace of creatine, a slight glycosuria and a slight increase in the output of total acetone. The appearance of the creatine was checked by the diacetyl test.

Exp. IV. A repeat experiment on the carbohydrate-rich diet but without the preliminary dose of morphia was carried out. The administration of the anaesthetic was continued for 150 minutes from 3 p.m. to 5.30 p.m.

TABLE IV. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	N. in g. T.N.	Urea	NH_3	Creatinine	Creatine	Sugar in g.	Acetone and aceto-acetic acid in g.
v	1000	amph.	1006	2·28	1·44	0·280	0·141	—	—	—
vi	1240	,,	1003	2·31	1·58	0·229	0·130	—	—	—
vii	1100	,,	1006	2·34	1·51	0·246	0·131	—	—	—
viii	420	,,	1004	0·62	0·34	0·068	0·028	—	—	0·0007
	1000	,,	1009	1·71	0·97	0·112	0·103	0·011	2·5	0·0087
	1420			2·33	1·31	0·180	0·131	0·011	2·5	0·0094
ix	1120	acid	1003	3·28	2·34	0·389	0·138	—	—	0·0076
x	1220	amph.	1005	2·39	1·50	0·314	0·123	—	—	0·0059

Again there is no rise in the output of total nitrogen on the day of the experiment but quite a marked one on the day following. The fall in the output of ammonia is quite definite although not so marked as in Exp. III. There is a slight fall in the output of urea and the excretion of creatinine is not affected. The output of creatine and sugar is much more marked, as

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is also total acetone. The diacetyl test was again used to control the appearance of creatine.

Exp. V. Carbohydrate-poor diet. Without previous administration of morphia the animal was anaesthetised for 150 minutes from 2.30 p.m. to 5 p.m. Apart from slight vomiting after the anaesthetic there were no ill effects of the ether.

TABLE V. (*Fat diet.*)

Day	Amount in cc.	Reaction	Specific gravity	N. in g. T.N.	Urea	NH_3	Creatinine	Creatine	Sugar in g.	Acetone and aceto-acetic acid in g.
iv	800	acid	1011	2·64	1·65	0·179	0·116	—	—	0·0124
v	1060	,,	1010	3·12	1·93	0·220	0·125	—	—	0·0113
vi	1000	,,	1010	2·80	1·68	0·185	0·125	—	—	0·0097
vii	200	,,	1005	0·37	0·23	0·024	0·016	—	—	0·0021
	1210	,,	1007	2·54	1·65	0·214	0·106	0·007	—	0·0117
	1410			2·91	1·88	0·238	0·122	0·007	—	0·0138
viii	1000	amph.	1009	3·75	2·59	0·179	0·113	—	—	0·0135

The effect on the output of total nitrogen is again marked on the day following. In this experiment instead of a fall in the excretion of ammonia there is quite a definite rise on the day of anaesthesia. The output of creatinine is unaltered on the day of experiment but there is quite a marked fall on the day following. As regards the output of sugar none was detected. The output of creatine is a mere trace. There was a small but appreciable rise in the excretion of total acetone.

Thus in both sets of experiments, in which the modes of production of the glycosuria are very different, there is associated with the disturbance of the carbohydrate metabolism an output of creatine. There is no question in any of these experiments, with the possible exception of Exp. V, of the output of creatine being only an apparent output due to the presence of aceto-acetic acid interfering with the estimation of the preformed creatinine.

Conclusions.

1. The injection of adrenaline produces a glycosuria associated with which there is an output of creatine.

2. There is also a rise in the output of total nitrogen due to the injection of the adrenaline.

3. Ether anaesthesia is followed by a slight glycosuria and creatinuria.

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4. A rise in the output of total nitrogen occurs on the day following the anaesthesia.

5. The output of creatine in these experiments is not "apparent," due to the presence of aceto-acetic acid, but is a real output.

These experiments were carried out at the suggestion and under the direction of Dr E. P. Cathcart, to whom I offer my thanks. Dr Cathcart is responsible for the injections.

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XLIV. ACIDOSIS AND SOME OF THE FACTORS WHICH INFLUENCE IT.

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Introduction.

The excretion of acetone, aceto-acetic acid, and β-hydroxybutyric acid, to which the name of acidosis was applied by Naunyn, is of great interest to the physiologist, the pathologist and the clinician. Its importance lies in the fact that the output of these substances affords us practically the only clue to the faulty metabolism of fat. It is true that a certain amount of information regarding the quantitative metabolism of fat has been acquired by a study of the respiratory quotient, but by this method we gain little information concerning the qualitative or intermediary metabolism. All that has been ascertained with certainty is that fat is, under normal circumstances, completely oxidised to carbon dioxide and water.

It has long been known that acetone is a normal constituent of the body fluids. It can be detected in small quantities in the blood, in the saliva, in the breath, in the liquor amnii and in the urine. Of these channels of excretion, that by way of the urine is of the most importance and the estimation of the urinary acetone has been adopted here. The other acetone bodies, namely aceto-acetic acid and β-hydroxybutyric acid, are also mainly found in the urine and some account of them will be given in our experiments.

One of the great questions which has been long under discussion [*vide* Magnus Levy, 1908; v. Noorden, 1907; Geelmuyden, 1900; Forssner, 1909, etc.] is the source of the acetone bodies, some workers maintaining that they were derived from fats alone, others just as actively supporting the contention that proteins played a part in their production. Modern work has shown,

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and the present communication will bear out the conclusion, that fats may be considered the main source, though, of course, it must be remembered that Dakin [1912] and others have clearly demonstrated that certain constituent amino-acids of the protein molecule can, on decomposition, yield aceto-acetic acid.

Methods.

The total nitrogen was estimated by Kjeldahl's method and the ammonia by Folin's method. The preformed acetone was estimated by Folin's method, using an apparatus modified to suit the occasion. The total acetone (acetone + aceto-acetic acid) and β-hydroxybutyric acid were estimated by Shaffer's [1908] method. Several workers have investigated this method of Shaffer's and all agree that when estimating the amount of acid in the sodium salt 90–96 % of the theoretical value is found. Kennaway [1914] also finds a similar value but is not satisfied that such a small error is present when the method is applied to urine. He states that "it is of course much more difficult to find out whether the acetone obtained from urine by oxidation is derived wholly from β-hydroxybutyric acid and there seems no satisfactory way of doing this." Certain variations in the results obtained by this method in previous experiments aroused some suspicions as to the possibility of a fallacy, so that before the present experiments were proceeded with, a series of control analyses was made. It was found for instance, when estimating total acetone in a normal urine of a subject on constant normal diet, that the amount of acetone varied greatly from day to day, and, as different quantities of basic lead acetate solution were added to each urine, according to the variations in the quantity of urine, the influence of this salt was naturally investigated first. Accordingly, dry basic lead acetate was heated in a test tube which was connected with another tube containing a measured volume of an alkaline solution of N/10 iodine. After heating 2 g. of the salt for a couple of minutes and then titrating the iodine solution in the ordinary way it was found that some substance capable of combining with iodine to the extent of 3·6 cc. N/10 iodine had been distilled off. In another experiment 10 cc. basic lead acetate solution (U.S.P.) were put into a test tube, 1 cc. H_2SO_4 added, as in estimation of acetone ($PbSO_4$ was precipitated), and the tube connected up as before with another test tube containing a measured volume of N/10 iodine in alkaline solution. The lead acetate tube was boiled for about a minute and some substance was given off which caused a loss of 0·8 cc. N/10 iodine. In view of the fact

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that one of the ordinary laboratory methods for the preparation of acetone is the dry distillation of calcium acetate, it may be assumed that in the present instance the iodine-combining substance distilled off was acetone. This assumption was confirmed by a positive result with the nitroprusside test.

Several other blank determinations were then carried out by the ordinary Shaffer method, but using water in place of urine.

40 cc. basic lead acetate solution (U.S.P.) were added to 250 cc. of water and the whole well shaken. 20 cc. ammonium hydrate were added, mixed, made up to 500 cc. and again well shaken. After standing for some minutes the liquid was filtered. 200 cc. of the filtrate were made up with water to 600 cc. and 15 cc. pure sulphuric acid added. A second 200 cc. were treated the same way. These were then distilled, using an ordinary condenser, until 200 cc. had collected in each receiving flask. The distillate was re-distilled, boiling for twenty minutes with 10 cc. 10 % sodium hydrate. Previous to each distillation the receiving flasks contained 300 cc. of cold water, and care was taken that the connecting tube dipped beneath the surface and that the water did not become hot. The acetone in the distillate was estimated by the iodine titration method. As the figures given show, a small quantity of acetone was evolved in each case.

N/50 iodine cc.	N/50 thiosulphate cc.	Difference cc.
5	3·2	1·8
5	3·3	1·7
5	3·05	1·95
5	3·35	1·65
4	2·25	1·75
4	2·4	1·6
2	0·5	1·5
2	0·7	1·3
4	2·2	1·8
4	2·05	1·95

The difference is seen to be fairly constant and to have an average of 1·7 cc. N/50 iodine.

When carrying out estimations in diabetic urine, or in urine containing a large quantity of acetone bodies, this error may be relatively small, but in normal urine a considerable error is always produced, in some cases more than 100 %.

When analysing normal urines—i.e. practically sugar free—10 cc. of the lead acetate solution were used if less than 100 cc. of urine were taken, 20 cc. solution for 100–150 cc. of urine and 40 cc. solution if more than 150 cc. of

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urine. Accordingly after the use of 40 cc. solution, 1·7 cc. was subtracted from the iodine difference obtained, and after 20 cc. or 10 cc. one-half or one-quarter of this amount.

The method, thus corrected, was found to be an excellent one for estimating even very small quantities of acetone.

In the estimation of β-hydroxybutyric acid Shaffer himself admits that there is an error of about 10 %.

Several check experiments were carried out, but, as the results obtained were so discordant, and as the other methods for the estimation of this substance were too slow for our purpose, β-hydroxybutyric acid was estimated in only a few of the experiments recorded in the present paper.

Excretion of Acetone by the Normal Individual[1].

A comparatively large number of investigations have been carried out from time to time on the normal urinary acetone output. The results which have been obtained vary to a certain extent, the differences being in part due to the differences in the method of estimation. The results which workers like Hirschfeld [1895] and Geelmuyden [1900] have obtained show that during the twenty-four hours 10–30 milligrams of total acetone are excreted in the urine. The results which I have found in the case of several healthy individuals are in complete agreement with these. It was found also, that on a constant diet the total acetone excreted is practically constant in amount. The quantity found is influenced by the diet, being greater with a high protein intake than with a low one. In one experiment of four days duration, where 400 g. of protein (mainly consisting of caseinogen) per diem were taken, 53 mg. of acetone + aceto-acetic acid were found in the fourth day's urine. The total nitrogen in the same urine was 52·16 g., and in the faeces 2·56 g.

In another experiment where a constant "standard" diet was taken, containing 118 g. of protein, derived from milk, eggs and bread, the amount of total acetone excreted daily in the urine lay between 15·7 mg. and 18·2 mg. The lowest recorded output of acetone in my own case was 5·2 mg. found on the third day on a diet of 100 g. cane sugar per diem.

While carrying out some investigations on post-anaesthetic diaceturia some very low total acetone outputs were found. One patient, who had been operated on for appendicitis, under chloroform and ether, excreted only

[1] The subject employed in all the experiments detailed in this paper was the writer himself.

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29·7 mg. total acetone during the four days following the anaesthetic, while another patient, who exhibited no post-anaesthetic diaceturia, excreted 5·1 mg. on the fourth day after a general anaesthetic.

Another point of some interest arises, namely whether, as in the case of the nitrogenous products of metabolism, there is a diurnal variation in the excretion of these acetone bodies. In order to elucidate this point the following experiment was carried out. The standard diet detailed below was taken for five consecutive days. On the first day, the food was taken in three equal parts, at intervals of eight hours. On the second day, two-thirds of the food was consumed in equal amounts two hourly from 7 a.m. till 11 p.m. At 11 p.m. the remaining third of the food was taken. On the third, fourth and fifth days one-twelfth of the food was taken two hourly, day and night. The urine was also collected two hourly and the two hourly output of total acetone and also of total nitrogen for the third, fourth and fifth days are given in the following table (Table I).

Diet.

Wheaten bread	360 g.	Protein	107 g.
Dried separated milk ...	252 „	Carbohydrate	480 „
Walnut butter	72 „	Fat	73 „
Cane sugar	211·2 „	Energy value	3081 cal.

It is seen that the rate of excretion of the acetone bodies is not absolutely constant from hour to hour, but that any variation detected cannot be related to any particular period of the twenty-four hours. Forssner [1909] obtained a very similar result.

Influence of the Nature of the Diet.

Another factor of very considerable importance is the actual nature of the diet. It has long been recognised that, when the external supply of carbohydrate is greatly diminished or entirely cut off, as in starvation, the condition known as acidosis rapidly ensues. The less carbohydrate there is metabolised, relative to the fat metabolised, the greater will be the degree of acidosis and the more acute its onset.

In the following experiments, the influence of fat and protein on the output of preformed acetone and of total acetone on a standard diet was investigated. The standard diet (see previous experiment) was taken for four days. The food was taken every four hours throughout that time. The urine was collected four hourly during the last three days. At 8 a.m. on the third day 300 g. of pure olive oil were taken in addition to the other

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TABLE I.

Day of diet	Hour	Total acetone mg.	Total nitrogen g.
3	7–9 a.m.	1·76	1·43
	9–11 „	1·68	1·43
	11–1 p.m.	1·35	1·25
	1–3 „	1·66	1·34
	3–5 „	1·82	1·29
	5–7 „	1·95	1·26
	7–9 „	1·73	1·20
	9–11 „	2·53	1·14
	11–1 a.m.	1·95	1·23
	1–3 „	1·98	1·32
	3–5 „	2·18	1·31
	5–7 „	1·40	1·51
4	7–9 „	1·37	1·62
	9–11 „	1·64	1·62
	11–1 p.m.	1·37	1·34
	1–3 „	1·85	1·41
	3–5 „	1·73	1·13
	5–7 „	1·98	1·50
	7–9 „	1·56	1·39
	9–11 „	1·58	1·43
	11–1 a.m.	1·72	1·28
	1–3 „	1·45	1·52
	3–5 „	1·79	1·55
	5–7 „	1·66	1·58
5	7–9 „	1·63	1·51
	9–11 „	1·81	1·65
	11–1 p.m.	1·43	1·63
	1–3 „	1·24	1·53
	3–5 „	1·31	1·57
	5–7 „	1·24	1·49
	7–9 „	1·47	1·39
	9–11 „	1·40	1·44
	11–1 a.m.	1·19	1·35
	1–3 „	1·23	1·29
	3–5 „	1·16	1·64
	5–7 „	1·63	1·79

food. The oil was well emulsified with water containing 3 g. anhydrous potassium carbonate. The urine collected at midnight and at 4 a.m. was examined in the morning and the remainder examined as collected.

The experimental data given below (Table II) show that the catabolism of fat in the presence of an abundance of carbohydrate does not produce any increase in the output of the acetone bodies in the urine, but rather a slight temporary decrease. This is probably due to the protein-sparing action of the fat, as indicated by the fall in the total nitrogen. As protein is responsible for some of the acetone present, it might be assumed that

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a diminished protein metabolism would result in less total acetone being formed. It will be noted that the decrease in the output of the acetone bodies was most marked in the case of the preformed acetone.

TABLE II.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
2	4–8 a.m.	2·15	1·63	75·6	2·59
	8–12 noon	2·88	1·63	56·4	2·90
	12–4 p.m.	2·64	1·97	74·8	2·55
	4–8 „	2·25	0·81	36·1	2·59
	8–12 m.n.	2·78	—	—	2·76
	12–4 a.m.	2·39	1·16	48·5	2·58
3	4–8 „	2·39	0·99	41·3	2·60
	8–12 noon*	2·44	0·99	40·4	2·70
	12–4 p.m.	1·93	1·39	72·0	2·52
	4–8 „	2·20	0·28	12·7	2·16
	8–12 m.n.	1·96	0·52	26·6	1·88
	12–4 a.m.	2·30	0·87	37·8	2·32
4	4–8 „	2·35	0·52	22·3	2·43
	8–12 noon	2·30	0·58	25·2	2·65
	12–4 p.m.	1·79	0·46	26·5	2·35

* 300 g. olive oil at 8 a.m.

When protein is added to the standard diet in place of the oil a definite increase of the total acetone results (Table III).

TABLE III.

Day	Hour	Total acetone mg.	Total nitrogen g.
3	7–11 a.m.	3·72	2·56
	11–3 p.m	3·48	2·37
	3–7 „	3·60	2·32
	7–11 „	3·99	2·32
	11–3 a.m.	3·14	2·16
	3–7 „	4·08	2·25
4	7–11* „	3·96	3·54
	11–3 p.m.	4·93	4·65
	3–7 „	4·62	3·88
	7–11 „	3·29	3·34
	11–3 a.m.	3·75	3·22
	3–7 „	4·20	3·08

* 100 g. caseinogen at 7 a.m.

The standard diet was taken for four days, during the first two days eight hourly, and during the next two days four hourly, the food being equally partitioned as in previous experiments. At 7 a.m. on the fourth day 100 g. of caseinogen were taken along with the usual food. A sample of the caseinogen boiled with dilute hydrochloric acid and filtered did not reduce Fehling's solution.

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Unfortunately, on this occasion the preformed acetone was not estimated, and it is therefore impossible to say which of the acetone bodies increased, or whether both were raised in amount. The figures are in keeping with those of other experiments, and all indicate that the greater the protein break-down, the higher is the amount of total acetone excreted.

Effect of Preceding Diet on the Acidosis of Starvation.

The next series of experiments was carried out chiefly to observe the effect of the preceding diet on the acidosis of starvation. This acidosis is almost certainly due to the great deficiency in the supply of carbohydrate available for immediate use. The sudden increase in the urinary acetone bodies probably occurs when the stored carbohydrate has all been utilised. Almost every experiment points to the fact that the animal organism prefers to derive its energy from carbohydrate rather than from fat, or even from protein. Carbohydrate is the most easily oxidised food-stuff and is a better protein sparer than fat, and for these reasons, when the body is starving and requiring the maximum amount of energy with the minimum of protein destruction, carbohydrate will be used so long as it is available. When this has been exhausted, most of the energy would appear to be derived from fat. The ratio of the fat oxidised to the carbohydrate oxidised then becomes much higher than it was previously, and, when this ratio rises above a certain definite value, abnormal quantities of acetone bodies appear in the urine. The results of experiments done on this subject point to the existence of a quantitative relationship, which has a very definite minimal value, between the fat and the carbohydrate utilised to prevent acidosis. In the light of these observations the initial rise in the excreted acetone bodies is readily understood.

Starvation following a high Carbohydrate Diet.

Second day before the fast—normal diet to which was added cane sugar and other carbohydrate-rich food.

Pre-day's diet consisted of:

Cane sugar	...	450 g.	Cornflour	...	40 g.
Tapioca	...	115 g.	Milk ...	...	50 g.
Bread ...	...	300 g.	Butter ...	...	15 g.
Potatoes	...	150 g.	Lean meat	...	50 g.

Some jam.

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This gives a carbohydrate intake of some 757 g. The urine was collected from the time at which the last meal was taken. None of the urine collected reduced Fehling's solution, though that on the pre-day did so slightly. The period of fasting was three days. The results are shown in Table IV.

TABLE IV.

Day of fast	Total acetone mg.		Total nitrogen g.
1	20·51		4·17
2	28·79		4·41
3	138·96	First 12 hrs 30·22 Second „ 108·74	—

Starvation after high Protein Diet.

Second day before—high protein diet

Pre-day:	Caseinogen ... 460 g.		Bread ...	150 g.
	Lean meat ... 100 g.		Butter ...	q.s.
		2 eggs.		

The last meal consisted of 120 g. caseinogen and the urine was collected from that time. The period of fast was three days (see Table V).

TABLE V.

Day of fast	Total acetone mg.		Total nitrogen g.
1	26·75		34·72
2	77·84		15·47
3	235·85	First 12 hrs 57·58 Second „ 178·27	18·92

Starvation after high Fat Diet.

Pre-day: 300 g. pure olive oil well emulsified with water and 3 g. K_2CO_3.
1 egg and 30 g. oatmeal at 10 a.m.
90 g. lean meat at 1 p.m.

Last meal, consisting of 150 g. olive oil, at 6 p.m. and the urine collected from that time. The period of fast was two days (see Table VI).

TABLE VI.

Day of fast	Total acetone mg		Total nitrogen g.
1	61·70		7·21
2	259·01	First 12 hrs 98·30 Second „ 160·71	10·53

These results show conclusively that the acetone output in the early days of starvation *is* governed by the nature of the preceding diet.

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In the first experiment there was sufficient stored carbohydrate to prevent acidosis for some forty-eight hours. The output of total acetone during that period was not above the normal. During the third day, however, a marked increase of the bodies in question occurred, 30·2 mg. being excreted in the first twelve hours and 108·7 mg. in the second. It will be noticed that the onset of acidosis is very rapid. This is found to be the case in almost every experiment, and, as will be shown later, its disappearance is even more rapid. This rise in the total acetone may occur at any part of the twenty-four hours, neither day nor night having any influence over it.

The second experiment shows that a large intake of protein previous to starvation also delays the onset of acidosis. The delay is not due to the presence of protein itself, for this is rapidly deaminised after absorption, the nitrogen-free part of the molecule being catabolised or stored, probably as carbohydrate. In all probability it is the carbohydrate derived from protein which postpones acidosis, and, as there would be less carbohydrate stored up at the beginning of this fast than at the beginning of the previous one, acidosis would here be apparent at an earlier period. On the first day's starvation 26·75 mg. of total acetone were found in the urine; but as the total nitrogen was 34·72 g. a considerable part of this acetone was almost certainly derived from the protein. On the second day the total acetone found was relatively much greater than the normal. On the third day, especially in its latter half, a further and more pronounced increase took place.

As one would expect, when a high fat diet precedes starvation, there is an increased output of acetone bodies almost from the beginning, due to the fact that this fat during its combustion would draw on the stored carbohydrate. The sudden rise in the total acetone excreted is here observed on the second day.

Acidosis in Starvation.

The amount of acetone excreted in the urine during starvation has been determined by Brugsch [1905], Bönniger and Mohr [1906] and others. On the first day the quantity is generally small. On the second day variable amounts are found, but by the third day a marked rise is always observed and the increase continues till about the fifth day. A diminution in the amount of the acetone bodies then occurs, and a more or less constant output of 0·4–0·8 g. is recorded, this being about half or less of the quantity found when the output is at its maximum.

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Brugsch for example found the following results for the twenty-third to the thirtieth day of a thirty days fast by Succi.

Day	Total acetone mg.	Total nitrogen g.
23	569	5·837
24	410	6·410
25	463	6·272
26	569	6·182
27	525	6·302
28	339	4·437
29	242	4·193
30	115*	8·421

* Lemonade with sugar taken.

To what is this fall in the total acetone output due? Acidosis can be diminished by the catabolism of carbohydrate or of protein, and if only for economic reasons, the former is the more likely cause of the change, unless some altogether unknown factor in the animal metabolism plays a part. We have next to find the source of this carbohydrate, assuming that it has brought about the decreased acetone excretion.

Landergren [1903] stated that the sole source of carbohydrate in starvation was protein. Now there is less protein metabolised per diem in the second week of starvation than in a like period during the first few days, and consequently at the later period less carbohydrate would be produced, leading to an increased fat : carbohydrate ratio. This would inevitably be accompanied by an accentuated acidosis. On the contrary, however, the reverse is found, so that another cause must be sought.

Although the conversion of fat or fatty acids into carbohydrate has not been proved, the possibility of such a change has been put forward by several physiologists of repute, and a certain amount of evidence in favour of it has accumulated. It does seem possible that, under special circumstances, when, for example, the body is in great need of carbohydrate, such a change may occur. That sugar can be derived from glycerol is already known, but whether this occurs in the body remains to be proved. A certain amount of evidence in favour of the view that in diabetes an increased output of sugar follows the administration of glycerol does certainly exist. If fat yields carbohydrate the diminished acidosis might readily be explained.

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Factors influencing Acidosis.

The results which have been dealt with up to the present deal with the factors which influence the onset of acidosis, and it is now proposed to consider some of the factors which influence the acidosis after it is definitely present.

1. *Effect of feeding Carbohydrate.*

The subject had been on a fat diet (320 g. pure olive oil per diem) for three days. At 7 a.m. on the fourth day 100 g. of cane sugar were taken and the same again at 2 p.m. At 6.30 p.m. and 11 p.m. eggs and bread and butter were taken.

Table VII gives the results for the third (the last) day of the oil diet and for the fourth day when carbohydrate was given.

TABLE VII.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
3	7–10 a.m.	89·83	11·84	13·1	1·94
	10–1 p.m.	85·72	11·60	13·5	1·70
	1–4 „	91·53	10·73	11·7	1·53
	4–7 „	88·87	11·84	13·3	1·49
	7–10 „	104·10	13·23	12·7	1·52
	10–1 a.m.	91·28	16·59	18·2	1·30
	1–4 „	114·49	18·22	15·9	1·76
	4–7 „	141·81	22·34	15·7	1·67
4	7–10* „	50·43	14·16	28·1	1·29
	10–1 p.m.	12·23	6·27	51·0	1·24
	1–4* „	9·96	5·57	55·9	1·19
	4–7† „	4·50	—	—	0·97
	7–10 „	2·27	1·86	81·8	1·21
	10–7† a.m.	4·98	3·48	69·9	3·60

Day	β-Hydroxybutyric acid
3	1273 mg.
4	206 „

* 100 g. of cane sugar at 7 a.m. and 2 p.m.
† Eggs, bread and butter at 6.30 p.m. and 11 p.m.

2. *Effect of feeding Protein.*

The subject had been fasting for two days, the fast being preceded by a high fat diet. At 6 p.m. on the second day 100 g. of caseinogen were taken, and no more food for twelve hours afterwards (see Table VIII).

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TABLE VIII.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
2	6–10 a.m.	50·19	7·35	14·64	1·88
	10–2 p.m.	53·33	7·54	14·15	2·20
	2–6* „	57·20	8·12	14·21	1·83
3	6–8 „	15·38	3·87	25·15	1·74
	8–10 „	6·91	3·19	46·15	1·42
	10–2 a.m.	10·54	2·71	25·68	4·28
	2–6 „	15·38	2·71	17·61	3·46

* 100 g. caseinogen at 6 p.m.

As will be seen from the preceding tables both carbohydrate and protein cause a rapid diminution in the amount of acetone bodies found in the urine.

(1) The sudden disappearance of acidosis after feeding sugar is very striking, the output of total acetone dropping from 141·8 mg. in one period of three hours to 50·4 mg. in the subsequent three hours. This change actually occurred even more rapidly than the figures indicate, since some thirty minutes would elapse before the sugar was available in the body, and during this time acetone bodies would be excreted at the same rate as before the sugar was taken. Evidently the presence of carbohydrate causes a profound alteration in the course of fat catabolism. The suddenness of the change suggests that it is the latest stages in the oxidation of the fatty acids which are affected. It is not improbable that the first stages in the oxidation of fat are accomplished independently of carbohydrate, but that the later stages of the catabolism are diverted from the normal in the absence of this material. At least the glyceryl radicle of fat can be oxidised in absence of carbohydrate.

The diminished acetone output was accompanied by an immediate fall in the total nitrogen, so that presumably the same moiety of carbohydrate which is available for the prevention of acidosis acts also as a protein sparer.

(2) As both absorption and combustion of protein take place more slowly than they do in the case of carbohydrate, the effect of protein on the metabolism is longer in becoming apparent. The acidosis was quickly checked, but not reduced to normal, probably because the caseinogen did not yield sufficient carbohydrate or did not yield it quickly enough.

The marked rise in the total nitrogen (about 100 %) in the first two hours after feeding showed that deamination was already going on.

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3. *Effect of feeding Fat.*

The subject had been fasting for three days. 200 g. pure olive oil, well emulsified, were taken immediately at the close of the fasting period. No further food was taken for twelve hours.

The effect of fat will be seen from the following table (Table IX).

TABLE IX.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
3	8–12 noon	27·68	3·68	13·28	1·89
	12–4 p.m.	38·12	4·64	12·18	2·02
	4–8 „	42·95	4·64	10·74	1·74
4	8–10* „	10·31	3·09	29·97	0·90
	10–12 m.n.	13·20	3·35	25·38	0·74
	12–8 a.m	91·81	19·34	21·07	—

* 200 g. olive oil taken at 8 p.m.

The figures show that after feeding fat, there was an immediate drop in the total acetone output during the first four hours and perhaps longer. This was succeeded by a very definite rise. A change was apparent during the first two hours so that the effect of the oil on the metabolism must soon have been felt. It must be remembered that the oil, being well emulsified, would be quickly absorbed.

More than one explanation is suggested for this unexpected decrease. The fat supplied certain energy needs which in the starving organism were probably covered by carbohydrate thus liberating carbohydrate for metabolic purposes. Again the oil might be more readily utilised than the body fat and consequently a sparing of the latter would occur, while the former would be broken down. If the first stage of this oxidation do not require the presence of carbohydrate in order to be normally carried out—and this is probably the case—then there will be a relatively greater amount of carbohydrate available to bring about the complete oxidation of the already partially broken down body fat, carbon dioxide and water being excreted in place of acetone bodies.

This decrease in the degree of acidosis, following immediately on taking fat, seems to be an argument in favour of the first stages in the oxidation of fat occurring without the aid of carbohydrate. A similar result will be alluded to in a future experiment. The subsequent rise in the total acetone output is again due to the disturbed quantitative relationship between the carbohydrate and the fat catabolised.

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4. *Effect of feeding Glycerol.*

In another experiment 100 g. of glycerol were fed at 8 p.m., after fasting for three days. The results obtained are given in Table X.

TABLE X.

Day	Hour	Total acetone mg.	Total nitrogen g.
3	8–12 noon	31·23	4·00
	12–4 p.m.	60·10	2·95
	4–8 „	86·93	1·88
4	8–10* „	37·86	1·15
	10–12 m.n.	12·47	0·90
	12–8 a.m.	5·42	5·04

* 100 g. glycerol were taken at 8 p.m.

Glycerol also causes a disappearance of acidosis. During the first two hours the decrease in the total acetone was slight, but by the end of four hours a marked diminution was observed. A normal amount of total acetone was excreted between the fifth and twelfth hours inclusive after taking glycerol.

How does glycerol act? It may possibly be converted into sugar. The greatly diminished acidosis which followed its ingestion suggests that such a conversion has taken place. This change may not occur under normal circumstances, but when carbohydrate is scarce such a conversion may be postulated. Again glycerol, as an energy yielding substance, will cause the body fat to be spared, thereby reducing the fat : carbohydrate ratio and bringing about a diminished acidosis. Such a marked change as was recorded could not have occurred, however, had glycerol acted only in this way.

5. *Effect of feeding Alcohol.*

The pre-day diet contained approximately 80 g. protein, 400 g. carbohydrate and 80 g. fat.

On each of the two following days 40 g. of absolute alcohol were taken and no other food. The alcohol was well diluted with water and taken at regular intervals from morning till night. Table XI shows the results obtained.

TABLE XI.

Day	Total acetone mg.	Total nitrogen g.	NH_3 g.
1	132·2	11·49	0·416
2	192·0	14·83	0·588

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The results confirm Hirschfeld's [1895, 1897] statement that alcohol does not prevent acidosis.

Being a source of energy it will probably cause a slight diminution in the total acetone by sparing fat, but it exerts no such influence as does carbohydrate, protein or glycerol on the later stages of the oxidation of fat.

Influence of high Fat Diet on Acidosis.

A series of experiments was next carried out to find the effect of protein-free fat-rich diets on the urinary output of acetone bodies. The results of two of these experiments are compared with results obtained in experiments where the fat was omitted from the diet and carbohydrate alone fed. Unfortunately, owing to the disagreeable nature of the diet and to the discomfort it produced, the experiments could not be carried out for longer periods than three days. At the beginning of each period, during which oil was being taken, 2–3 g. calcium carbonate were taken. Diarrhoea was altogether absent and, in fact, the bowels rarely moved. This is in marked contrast with the extremely severe diarrhoea met with on a fat diet, when butter is the fat ingested.

Pure Fat Diet.

The pre-day diet contained very little carbohydrate and consisted of 6 eggs, 320 g. filleted cod, 280 g. fresh haddock, 170 g. potatoes, 50 g. bread, butter, q.s.

Total carbohydrate about 84 g., the last of which was taken seventeen hours before the oil diet began.

Diet days. 80 g. pure olive oil, emulsified as before, using 1·5 g. potassium carbonate, were taken four times a day at equal intervals, viz. 7 a.m., 1 p.m., 7 p.m., 1 a.m. This was continued for three days. The urine was collected three hourly from 7 a.m. on the first day. The first table (Table XII) is a daily chart, the second (Table XII A) a three hourly one.

TABLE XII.

Day	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	β-Hydroxybutyric acid mg.	Total nitrogen g.
1	147·9	37·4	25·3	411	14·78
2	389·2	61·7	15·8	616	14·96
3	817·6	116·4	14·3	1273	13·50
Day after	84·4	32·1	38·1	206	9·51

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TABLE XII A.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
1	7–10 a.m.	2·25	—	—	2·57
	10–1 p.m.	4·01	0·81	20·2	1·94
	1–4 „	8·66	2·21	25·5	1·83
	4–7 „	22·35	6·50	29·0	1·43
	7–10 „	11·72	4·13	35·3	1·31
	10–1 a.m.	35·90	8·82	24·6	1·27
	1–4 „	35·32	8·12	22·9	1·88
	4–7 „	27·71	6·85	24·7	2·00
2	7–10 „	53·33	7·25	13·6	1·92
	10–1 p.m.	49·70	8·47	17·0	2·05
	1–4 „	52·85	7·89	15·0	1·82
	4–7 „	48·98	6·61	13·5	1·45
	7–10 „	41·73	6·15	14·9	1·32
	10–1 a.m.	34·72	8·47	24·4	1·59
	1–4 „	42·94	8·34	19·4	1·66
	4–7 „	64·94	8·53	13·1	1·87
3	7–10 „	89·83	11·84	13·1	1·94
	10–1 p.m.	85·72	11·60	13·5	1·70
	1–4 „	91·53	10·73	11·7	1·50
	4–7 „	88·87	11·84	13·3	1·49
	7–10 „	104·10	13·23	12·7	1·52
	10–1 a.m.	91·29	16·59	18·2	1·30
	1–4 „	114·49	18·22	15·9	1·76
	4–7 „	141·81	22·34	15·7	1·67

A gradual increase in the urinary acetone bodies is observed from beginning to end. It will be noticed, however, during the second and third days of the diet, that on most occasions there was less total acetone in the urine passed during the second three hours after taking oil, than during the first three hours. A similar occurrence was observed and commented on in a previous experiment where oil was fed during starvation.

How long the increasing acidosis would have continued it is difficult to say. Would it cease earlier or later when fat is being fed than when the body is living on its own fat? Would the acidosis become so great as to produce serious symptoms? These questions must remain unanswered till a more prolonged series of experiments is carried out. Is there a more pronounced acidosis when the body is living on food fat than when living on its own fat? Forssner [1910] found that the addition of fat to a diet of fat and protein caused a rise in the total acetone excreted. In the experiment at present under consideration there is a much greater acetone output than in any of the starvation experiments. Moreover the highest acetone output during starvation was found after a high fat diet, when the body was using

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external fat as well as its own fat, so that as far as these results go this question may be answered in the affirmative. Forssner gave as a reason for this either that food fat is broken down in a different manner from body fat or that part of the former is converted into acetone bodies after ingestion.

The preformed Acetone.

The figures in this experiment, as well as those of other experiments communicated in this paper, show that the greater part of the total acetone is present in the form of aceto-acetic acid. Where the amount of total acetone is small the percentage of preformed acetone is high, perhaps 50–60 or more, but where the total acetone output is high only about 15 % of it is in the form of preformed acetone. A similar result was obtained by Piper [1913] and led him to use the term diaceturia in preference to acetonuria.

It is not to be supposed that the small quantity of acetone found in the urine is all that is produced in the body. This substance is very volatile and is excreted in larger quantities in the breath than in the urine. In order to test this point the following experiments were carried out.

On the evening of the third day on this diet about half of the air expired during twenty minutes was passed through a measured quantity of alkaline N/50 iodine with the result that 1·5 mg. of acetone were detected. Owing to the lack of a proper respiratory apparatus it was impossible to carry out an absolute quantitative examination of the expired air, and the result obtained must be regarded as representing a mere fraction of the acetone present. The breath had a marked odour of acetone.

EFFECT OF ADDING CARBOHYDRATE TO A FAT DIET.

The addition of sugar to the diet causes a great reduction in the amount of acetone bodies excreted. The greater the weight of sugar added the less is the amount of acetone bodies found.

In order that the results might be uniform the quantities of protein and fat taken on the day preceding each of this series of diets were practically the same in every case, the former amounting to some 180 g. and the latter to about 80 g. The same quantity of carbohydrate was taken on each pre-day as was taken on the succeeding diet days. The olive oil was always emulsified as already described.

The addition of 60 g. cane sugar reduced the daily amount of urinary acetone from decigrams to centigrams, but the well-marked increase in the total acetone from day to day showed that a greater amount of sugar was

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required to prevent acidosis. With the addition of 100 g. of cane sugar there is still more acetone than normal after the first day. When 150 g. of sugar were added to the fat more acetone was excreted on the third day than on the first, but the increase was slight. The amount of cane sugar required to prevent acidosis when 320 g. of fat are fed is then about 150 g. If all the oil is utilised the fat: carbohydrate ratio will be a little more than 2 : 1.

TABLE XIII.

Diet: 60 g. cane sugar—320 g. olive oil.

Day	Total acetone mg.	β-Hydroxybutyric acid mg.	Total nitrogen g.
1	40·13	256	10·97
2	60·56	210	9·18
3	81·11	499	7·55

TABLE XIV.

Diet: 100 g. cane sugar—320 g. olive oil.

Day	Total acetone mg.	β-Hydroxybutyric acid mg.	Total nitrogen g.
1	15·37	173	11·54
2	35·01	268	10·85
3	69·72	234	4·83

TABLE XV.

Diet: 150 g. cane sugar—320 g. olive oil.

Day	Total acetone mg.	Total nitrogen g.
1	10·12	6·94
2	21·47	4·73
3	24·05	2·24*

* Repeated analysis showed this result to be correct.

Effect of feeding Carbohydrate alone.

When 30 g. of sugar per diem are taken the amount of acetone found is only one-sixth to one-eighth of that found when the organism is totally deprived of food (Table XVI).

TABLE XVI.

Diet: 30 g. cane sugar per diem.

Day	Total acetone mg.	β-Hydroxybutyric acid mg.	Total nitrogen g.	NH_3 g.
1	27·86	174	9·12	0·366
2	25·47	254	10·35	0·337
3	34·91	601	10·34	0·396

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With 60 g. sugar more acetone was found than in the previous experiment. This must be due to the effect of the preceding diet, though the pre-day diet was almost the same in each case (Table XVII).

TABLE XVII.

Diet: 60 g. cane sugar per diem.

Day	Total acetone mg.	β-Hydroxybutyric acid mg.	Total nitrogen g.
1	21·64	186	8·51
2	45·51	224	8·01
3	79·05	214	9·70

TABLE XVIII.

Diet: 100 g. cane sugar per diem.

Day	Total acetone mg.	Total nitrogen g.
1	6·6	6·94
2	9·7	11·28
3	5·2	9·81

When 100 g. of cane sugar are taken (Table XVIII) in the course of the twenty-four hours, there is less total acetone found in the urine than is found when one is taking a normal diet. This quantity of sugar is sufficient then to prevent acidosis when no fat is taken in the food. As there was not much work done, about 2500 cal. would supply sufficient energy for one day. The sources of energy would be roughly as follows:

100 g. cane sugar	380 cal.
9 g. N = 57 g. protein	235 cal.
	615 cal.

The remaining 1885 cal. would be mainly derived from 203 g. body fat. Here again the fat : carbohydrate ratio is a little more than 2 : 1. While it is freely admitted that these two experiments giving a similar fat : carbohydrate ratio are not absolutely conclusive, the assumption may be made that there is a definite minimum fat : carbohydrate ratio required to prevent acidosis, and that this ratio is a little more than 2 : 1.

Effect of the various Diets on the Total Nitrogen.

It was also noticed that the more sugar there was added to the diet the less was the amount of total nitrogen found in the urine, and that on the diets containing fat + carbohydrate less total nitrogen was eliminated than on the diets of carbohydrate alone. In each experiment where the diet consisted of olive oil a feeling of intense lassitude and dullness was present,

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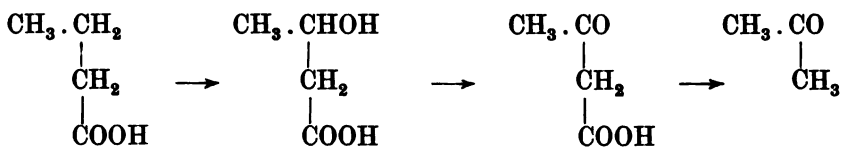
accompanied by abdominal discomfort. The former symptom was never absent, while the latter persisted for about two hours after taking oil. No vomiting occurred in any of the experiments recorded although a feeling of intense nausea was frequently present. A few experiments had to be discontinued owing to rejection of the oil even when taken through a stomach tube. These symptoms were less marked when sugar was added to the diet. If carbohydrate (100 g. cane sugar) was taken at the conclusion of the oil period all feelings of discomfort disappeared in less than two hours, although the limbs remained "tired" for some five hours longer. During these diets thirst was rarely experienced and little water was taken. Immediately after taking food an intense thirst developed and remained for about a day.

The significance of the Acetone Bodies.

It is known that in the body the fatty acids are oxidised in the β-position, and that eventually butyric acid is always formed [Ringer, 1913].

The prevailing opinion is that the oxidation of this acid in the β-position gives rise to β-hydroxybutyric acid as a normal intermediary product of fat metabolism. In the absence of carbohydrate this acid is only partially oxidised, aceto-acetic acid and acetone being formed.



Blum [1910] however maintains that normally butyric acid is first converted into aceto-acetic acid and that this is reduced to β-hydroxybutyric acid. Marriot [1914] also favours this view. In any case when carbohydrate is available the fatty acids are completely oxidised, carbon dioxide and water being the end products.

How does this carbohydrate act?

Ringer [1913] concludes from a series of very interesting experiments that it is not the whole carbohydrate molecule, but only the CHO radicle, that is necessary to prevent acidosis. He found that gluconic acid, differing only from glucose in having the CHO radicle replaced by CO.OH, did not prevent acidosis, while $CH_3 . CHO$ and $C_2H_5 . CHO$, when fed to phloridzinised dogs, brought about a great decrease in the total acetone output.

Ringer [Ringer, Frankel and Jonas, 1913] has also shown that isobutyric acid, when fed to phloridzinised dogs, gives rise to extra glucose and

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diminished acidosis; whereas normal butyric acid does not give rise to glucose but to an increased acidosis. He suggests that a glucosidic union is accomplished between β-hydroxybutyric acid and glucose by means of the CHO radicle, and that the derivatives of normal butyric acid are in this way converted into iso-compounds. This would explain the seeming necessity of a constant fat : carbohydrate ratio.

But more than a mere chemical union between β-hydroxybutyric acid and glucose seems to be involved. This is suggested by the fact that the feeding of 30 g. of sugar is sufficient to reduce the total acetone of complete starvation from 200–300 mg. to 35 mg. (see Table XVI), while a further addition of 70 g. of sugar is necessary to reduce this to less than 10 mg. This reduction could not be due to a diminution of the quantity of acetone derived from protein, as the total nitrogen in the latter case was quite as high as in the former. Again, the addition of 60 g. of sugar to a fat diet reduced the total acetone from 818 mg. to 81 mg. (Tables XII and XIII) while with a further addition of 90 g. of sugar 24 mg. of total acetone were excreted.

Ringer, in his experiments already mentioned, administered acetaldehyde and propyl aldehyde to phloridzinised dogs on a constant diet. The result was that not only was the acetone in the urine greatly diminished, but there was an increase in the output of sugar. This increase was more than could be accounted for even if all the carbon in these aldehydes had been converted into glucose carbon. He concluded that this increase in glucose was due to some non-glucogenetic substance in the animal metabolism becoming glucogenetic owing to the influence of the CHO radicle. Perhaps the same change can take place when sugar is given in starvation. A small quantity of carbohydrate may be sufficient to bring about the necessary change, while a large amount may be unable to increase its extent, or, when the non-glucogenetic substance has become glucogenetic it may only remain in that condition so long as acidosis is present in a marked degree. It is very probable that this potential glucogenetic substance is either fat itself or one of the products formed during the early stages of fat catabolism.

This work was carried out under the direction of Dr E. P. Cathcart, to whom I offer my thanks.

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Conclusions.

1. The quantity of acetone bodies excreted by the normal individual, on an ordinary diet containing a sufficiency of carbohydrate, is influenced chiefly by the protein intake. On an ordinary diet 10–30 mg. are excreted daily.

2. The administration of quite small amounts of carbohydrate to the starving organism brings about a great reduction in the acidosis.

3. The administration of protein to the fasting organism causes a similar decrease, but this is neither so marked nor so rapid as in the case of carbohydrate.

4. The administration of glycerol under similar conditions also causes a very definite reduction in the acidosis.

5. The administration of fat to the starving organism increases the acidosis.

6. Administration of alcohol is without effect on the degree of acidosis.

7. The amount of acetone bodies in the urine during the first few days of starvation depends on the initial carbohydrate storage. These substances appear in abnormal amounts immediately the ratio of the fat to the carbohydrate burnt becomes greater than about 2 : 1. Immediately the ratio becomes less than this the acetone output is reduced to normal.

8. While a relatively large amount of carbohydrate is required to prevent acidosis, quite a small amount suffices to check it very markedly.

9. When acetone bodies are excreted in excessive amounts they are for the most part derived from fat.

10. Some evidence is put forward in favour of the possibility of fat being converted into carbohydrate in the body.

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XLV. THE FIXATION OF SALVARSAN AND NEOSALVARSAN BY THE BLOOD AFTER INTRAVENOUS INJECTION.

By WILLIAM JOHN YOUNG.

From the Australian Institute of Tropical Medicine, Townsville.

(*Received October 27th, 1915.*)

It was found by Nierenstein [1908] that when serum was shaken with atoxyl (sodium *p*-aminophenylarsenate) and the proteins subsequently precipitated from the mixture with tannic acid, the precipitate invariably contained arsenic which could not be removed by washing. He further observed that when derivatives of atoxyl were used instead of atoxyl itself, arsenic was present in the precipitate only in those cases in which the derivative contained a free amino or imino group, whereas negative results were obtained with compounds such as benzoyl-acetyl-atoxyl, in which both hydrogens of the amino group were substituted, and with sodium *p*-hydroxyphenylarsenate, in which the amino group of atoxyl is replaced by hydroxyl.

In each case in which arsenic was present in the tannic acid precipitate the "atoxyl serum" could not be freed from arsenic by continued dialysis.

Breinl and Nierenstein [1908, 1909, 1 and 2] found that the same occurred when atoxyl and its derivatives were injected into animals. Arsenic was found in the serum only after the injection of compounds containing free amino or imino groups. It was concluded from these experiments that atoxyl combines with the serum proteins by means of its amino group.

The experiments described below were performed to ascertain whether similar combinations could be obtained with salvarsan and neosalvarsan. As these compounds are much less stable in solution than atoxyl, it was not possible to carry out any experiments in vitro, and only experiments with living animals were made.

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For the detection of arsenic, the serum was decomposed with a mixture of sulphuric and nitric acids, all trace of nitric acid removed by continued evaporation, and the residue tested for arsenic by the modification of Gutzeit's method recommended by Sanger and Black [1907]. Care was taken to employ reagents containing no arsenic.

The Fixation of Salvarsan and Neosalvarsan by Serum.

Goats were injected intravenously with salvarsan or neosalvarsan and blood was drawn after various intervals of time. The blood was allowed to clot and the serum poured off and filtered. The serum was then dialysed in parchment bags against running water or in some cases against running saline to prevent precipitation of the globulins.

Dialysis was continued until all dialysable arsenic was eliminated from the serum, which was ascertained by dialysing for 12 hours against a litre of distilled water or saline, evaporating the dialysate to a small bulk and testing for arsenic.

The dialysed serum was then filtered and 50 cc. decomposed and tested for arsenic. In every case arsenic was found in the serum. In some cases a portion of the dialysed serum was precipitated with 2 % tannic acid solution, the precipitate was well washed and decomposed and tested for arsenic with a positive result.

These experiments show that after injections of salvarsan and neosalvarsan, some arsenic is bound to the serum proteins in a form which cannot be removed by dialysis.

A control experiment was performed in which a very dilute solution of neosalvarsan was dialysed into running water for some days and the residue, on decomposing, was found to contain no arsenic.

It was found that continued dialysis was necessary before the dialysate was obtained free from traces of arsenic, and in some cases traces dialysed out even after more than seven days. It seemed probable that the combination with the serum slowly decomposed.

(1) *Goat* 1. Injected intravenously with 0·3 g. salvarsan. After 24 hours it was bled, the serum separated and 80 cc. dialysed as above.

Serum before injection—arsenic absent.

After injection 40 cc. dialysed serum—arsenic present; 40 cc. precipitated with tannic acid—arsenic present in ppt.

(2) *Goat* 2. 0·9 g. neosalvarsan was injected intravenously and serum obtained after 7 hours and 24 hours; both dialysed as before.

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Serum before injection—arsenic absent.

7 hours after injection—tannic acid ppt. from 50 cc. serum—arsenic present.

24 hours after injection 50 cc. serum used—arsenic present.

(3) *Goat* 3. 0·75 g. neosalvarsan injected intravenously, bled after 12 hours and serum dialysed.

Serum before injection 50 cc.—arsenic absent.

Serum after injection 50 cc.—arsenic present.

(4) *Goat* 4. 0·8 g. neosalvarsan injected intravenously, bled after 16 hours and serum dialysed.

Before injection 50 cc. serum—arsenic absent.

After injection 50 cc. serum—arsenic present.

An experiment was also performed to see if inorganic arsenic was bound in the serum in a similar manner. A goat was injected intravenously with an amount of arsenious acid corresponding with 0·6 g. neosalvarsan, dissolved in sodium hydrate and diluted with saline. After 24 hours blood was drawn, and the serum dialysed. 100 cc. of serum after dialysis gave no arsenic test. The same goat was subsequently injected intravenously with 0·6 g. neosalvarsan and bled after 24 hours. The serum after dialysis gave a positive arsenic reaction.

Time required for the arsenic to be completely eliminated from the blood.

Many investigations have been made on the rate of elimination of arsenic from the body after injections of salvarsan. Amongst the more recent may be mentioned that of Beveridge and Walker [1911], who found that arsenic could be detected in the urine of rabbits up to the eleventh day after intravenous injection of salvarsan and neosalvarsan. Stümpke and Siegfried [1911] claimed that traces of arsenic were found in the urine of men and other animals even after several months had elapsed, but they were unable to demonstrate the presence of arsenic in the blood later than 24 hours after intravenous injection, and they suggested that secondary depôts of arsenic were formed in the organs and then gradually excreted in the urine.

Schütte [1912] stated that with horses no arsenic could be detected in the urine and faeces later than 13 days after intravenous injection of salvarsan.

Stühmer [1914] investigated the properties of serum drawn at increasing intervals after intravenous injection of salvarsan and neosalvarsan to ascertain how long after injection it was possible to detect specific therapeutic properties in the serum. He compared the different sera with regard to their protective

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action against trypanosome infection of mice, and at the same time tested the sera for the presence of free salvarsan by the Ehrlich-Bertheim reaction. The solution employed for this reaction is made by dissolving *p*-dimethyl-aminobenzene in concentrated hydrochloric acid, adding excess of mercuric chloride and dissolving any precipitate which forms by the addition of a few drops of hydrochloric acid. With this solution salvarsan gives an orange precipitate.

Stühmer found that there was a parallelism between the protective action of the sera against trypanosomes and the intensity of the Ehrlich-Bertheim reaction. Serum drawn as late as the seventh day after injection of salvarsan postponed the infection. This action was increased when the serum was heated to 56° C. and the intensity of the Ehrlich-Bertheim reaction was also increased. With neosalvarsan after the second day the serum gave no reaction either chemically or biologically. Thus after two days no free neosalvarsan appeared to be present in the serum.

Riebes [1914], employing Abelin's [1912] diazo reaction with resorcinol for detecting the amino group of salvarsan, found that in the majority of cases the reaction disappeared from serum three hours after the injection.

From these experiments it appears that free salvarsan and neosalvarsan are eliminated in a very few days from the blood.

The following experiments were made to ascertain how long a time after injection "bound arsenic" could be detected in the serum. The serum drawn at different intervals was tested for free salvarsan by the Ehrlich-Bertheim reaction, it was then dialysed into running water and the residue decomposed and tested for arsenic as before. It was found almost impossible to see the orange precipitate or coloration in the presence of the serum when very little neosalvarsan was present, the serum was therefore dialysed into water and the reagent added to the latter.

0·9 g. neosalvarsan was injected intravenously into a goat. 50 cc. serum were used in each case.

TABLE I.

Time after injection	As in dialysed serum	E.-B. reaction
7 hours	+	+
24 ,,	+	Faint colour
2 days	+	,,
6 ,,	+	–
19 ,,	trace	–
28 ,,	–	–

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After 19 days therefore a trace of bound arsenic was still detected in the serum, whereas a faint reaction for salvarsan was only obtained after two days.

In another experiment, Table II, 0·8 g. neosalvarsan was injected intravenously.

TABLE II.

Time after injection	As in dialysed serum	E.-B. reaction
16 hours	+	+
24 „	+	faint colour
48 „	+	–
6 days	+	–
21 „	trace	–

In this experiment a trace of arsenic was still found in the dialysed serum after twenty-one days.

From these results it is seen that the arsenic which is bound in the blood is only very slowly eliminated, whereas the bulk of the neosalvarsan is quickly excreted, only a trace being present after the first twenty-four hours.

Distribution of "bound arsenic" in the blood.

The following experiment was carried out to ascertain in what parts of the blood arsenic was retained.

A goat which had received 0·8 g. neosalvarsan was bled after 7 days so that all free neosalvarsan should be excreted. 150 cc. of blood were defibrinated, the red cells separated by centrifugalisation and repeatedly washed in the centrifuge with normal saline. The final washings from the red cells were found to be free from arsenic. The fibrin after washing was free from arsenic. Arsenic was present in the red blood cells and also in the plasma after dialysis.

Summary.

After intravenous injection of goats with salvarsan and neosalvarsan, the serum contains arsenic in a form which cannot be separated from the proteins by dialysis, and which is precipitated with the serum proteins by tannic acid. Salvarsan and neosalvarsan behave, therefore, in a similar manner to atoxyl.

No such combination is obtained when inorganic arsenic is injected.

This combined arsenic is found in the blood long after all free salvarsan and neosalvarsan have been eliminated.

This combined arsenic is found in the plasma and in the red blood cells, but no trace of arsenic is retained in the fibrin.

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XLVI. THE EFFECT OF INGESTION OF UREA, SODIUM LACTATE AND SODIUM BICARBONATE ON THE REACTION OF THE BLOOD AND THE COMPOSITION OF THE ALVEOLAR AIR IN MAN.

By GORO MOMOSE.

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(*Received October 29th, 1915.*)

The present paper records an attempt to test the question whether certain substances taken by the mouth have the effect of making the blood in the body more alkaline.

Some preliminary experiments on the subject were made by Wolf and Barcroft [1915], who in a few experiments on dogs and on Barcroft himself found that blood exposed to oxygen at 17 mm. pressure took up somewhat more of the gas after ammonium citrate or urea had been taken. This suggested increased alkalinity of the blood.

Toyojiro Kato [1915, 1] recently observed that addition of certain alkaline substances to the blood, NaOH, Na_2CO_3, $NaHCO_3$, Na_2HPO_4, accelerates the oxidation and retards the reduction of blood. Accepting this, one would expect that alkali would increase the percentage saturation of oxygen in blood exposed to a given oxygen pressure, just as acid reduces the percentage saturation [Barcroft and colleagues, 1914]. We have then used this measurement as a test for the changed reaction.

The method of exposing blood to a standard gas mixture is that described by Kato [1915, 2], in which 1–2 tenths of a cc. is put into the bulb of a small pipette, gas is then passed over it from a gas holder, the pipette being rotated in a water bath at 37°–38° C. for about ten minutes. This suffices to secure

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complete equilibrium between the blood and the gas. The blood is then transferred to the differential gas analysis apparatus designed by Barcroft [1914, p. 300] for the analysis of 0·1 cc. of blood. In this its percentage saturation is determined.

On account of the novelty of Kato's tonometer I thought it desirable to test the method rigorously. The following are the results of two sets of determinations in which I tried to see whether on repeated analysis the same blood yielded similar values for the percentage saturation.

Percentage Saturation of Oxygen in Blood.

No. of exp.	Number of determinations	Animal	Maximal result	Minimal result	Mean
I	7	Sheep	58·8	53·3	55·3
II	7	Rabbit	43·8	39·8	41·1

To these results I added some on the percentage saturation of Barcroft's normal blood when exposed to 17 mm. O_2 pressure. They are as follows:

Experiment III.

	Percentage saturations				
(1)	77·1	75·1	79·6	—	mean 77·3
(2)	74·5	71·7	67·8	—	„ 71·3
(3)	71·0	74·0	73·4	73·9	„ 73·8
(4)	67·6	67·6	71·7	—	„ 69·1

These results shew that, so far as the method of gas analysis is concerned, any individual sample of blood gives very concordant results. They suggest that the blood of the same person varies to a trifling extent from day to day. This result must however be received with great caution, lest it should be due to a slight want of uniformity in the gas used on different occasions.

Results on my own blood were slightly more uniform:

Experiment IV.

	Percentage saturations		
(1)	72·8	72·5	mean 72·7
(2)	73·1	69·6	„ 71·4
(3)	74·0	72·1	„ 73·0

In no case do the above observations include cases in which substances for investigation such as urea, sodium lactate etc. have been taken by the patient so recently as the day before the observation.

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Experiments on the percentage saturation of human blood at 17 mm. O_2 pressure.

The following experiments shew that urea taken by the mouth causes the blood to become more alkaline, i.e. causes the percentage saturation in the blood to rise when exposed to the standard mixture.

Effect of Urea on Percentage Saturation of Blood.

		Percentage saturation				
			After			
					Approximate time	
No. of exp.	Dose	Before	1 hour	2 hours	5 hours	Next day
V	15 g. urea in 250 cc. water	77·3	82·0	—	84·2	—
VI	"	71·3	—	—	76·7	72·0
VII	"	73·1	—	80·2	—	—

Poulton and Ryffel [1913], who performed experiments on the percentage saturation of blood in uraemic patients, state that the addition of urea, in small concentrations, to blood does not change its affinity for oxygen. This I verified:

Experiment VIII. Rabbit's Blood.

	Urea added to 1 cc. blood	Percentage saturation with oxygen at 17 mm.	
(1)	Nil	48·8	Mean of 2 observations
(2)	0·1 cc. N/10 CON_2H_4	48·1	" " "
(3)	0·2 cc. "	47·1	" " "
(4)	0·3 cc. "	49·4	" " "

The solution of urea was normal in reaction. Since the urea itself does not affect the affinity of blood for oxygen, the inference is that urea taken by the mouth becomes partially converted into ammonia in the body [Wolf, 1912].

Exp. IX. It therefore became of interest to ascertain the concentration of ammonia, which, if added directly to blood, would produce the same change as the urea which was given by the mouth. This proved to be 0·2 cc. of N/10 NH_3 per cc. of blood or 0·017 %. This quantity changed the percentage saturation at 17 mm. from 73 to 83·7 %. (Mean of two determinations: 81·8 and 85·5.)

Sodium lactate was suggested by Sir Almroth Wright on the ground of its rapidly causing the urine to become alkaline. It does not follow that

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the blood would become appreciably alkaline for the sodium might be rapidly eliminated.

The preparation of sodium lactate which we used was slightly acid in reaction and when added to defibrinated blood it caused the percentage saturation at 17 mm. O_2 to drop, when added in dilute solutions; after 0·2 cc. N/10 lactate further addition had no effect, presumably because the degree of ionisation does not increase.

Experiment X. Rabbit's Blood.

	Sodium lactate added to 1 cc.	Percentage saturation with oxygen at 17 mm.	
(1)	Nil	48·8	Mean of 2 determinations in each case
(2)	0·1 cc. N/10 $NaC_3H_5O_3$	41·8	" " "
(3)	0·2 cc. " "	32·2	" " "
(4)	0·3 cc. " "	32·9	" " "

Given by the mouth however sodium lactate has precisely the opposite effect.

Experiments XI, XII and XIII. Effect of Sodium lactate.

No. of exp.	Dose	Percentage saturation with oxygen: Before	After: 1 hour	2 hours	3 hours	4 hours	Next day
XI	5 g. in about 10 cc. of water	72·7*	74·7	83·9	78·6	—	—
XII	"	74·4	75·5	—	77·5	72·5	72·6
XIII	"	72·6	76·2	88·0	74·1	75·0	—

* Each of these results is the mean of two determinations.

These figures indicate that the maximal effect is obtained at the end of about two hours. The question then arises, to how much alkali does this correspond? On this point I obtained the following data by the addition of NaOH directly to defibrinated blood.

Experiment XIV. Addition of NaOH to Human Blood (Barcroft).

	NaOH added to 1 cc. of blood	Percentage saturation with oxygen at 17 mm.	
(1)	Nil	69·3	Mean of 8 determinations
(2)	0·1 cc. N/10 NaOH	80·1	" 4 "
(3)	0·2 cc. "	80·7	" 3 "

The effect of adding urea, sodium hydroxide, sodium lactate and ammonia to defibrinated blood is shewn in Fig. 1.

A third substance which I tried was sodium bicarbonate.

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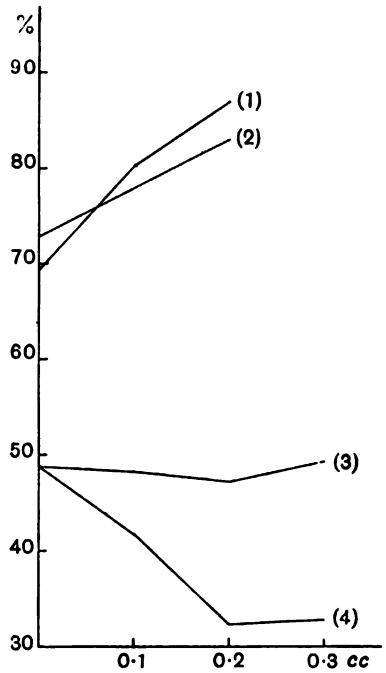

Fig. 1.

Ordinates = Percentage saturation of blood at 17 mm. oxygen pressure.
Abscissae = Quantity of drug added.

(1) N/10 NaOH (Blood of Barcroft)
(2) N/10 NH_3 („ Momose)
(3) N/10 CON_2H_4 (Blood of Rabbit)
(4) N/10 $NaC_2H_3O_2$ („ „)

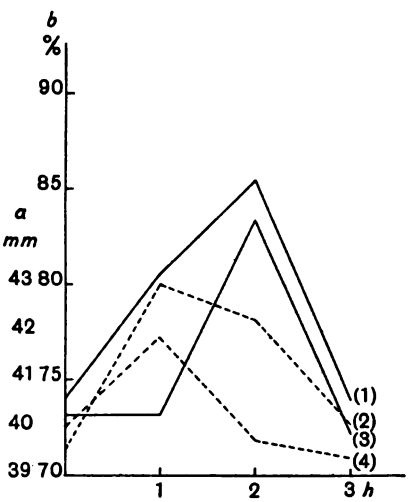

Fig. 2.
(3), (2). Exp. XVII.
(1), (4). Exp. XVIII.

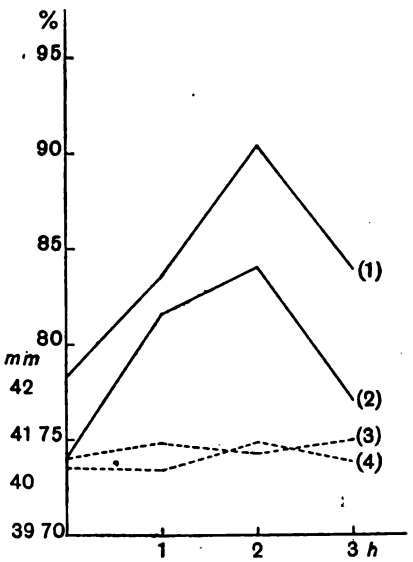

Fig. 3.
(1), (4). Exp. XIX.
(2), (3). Exp. XX.

Ordinates: (*a*) mm. pressure of CO_2 (dotted line).
(*b*) Percentage saturation of blood at 17 mm. oxygen pressure (continuous line).
Abscissae = Time after taking dose in hours.

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It might be expected that the addition of substances which would raise the percentage saturation at 17 mm. would also raise the alveolar carbonic acid. I made numerous measurements of the alveolar CO_2, therefore, before and after taking sodium lactate with the following results:

Alveolar CO_2 pressure in mm. (Momose).

No. of exp.	Dose of sodium lactate	Before	After 1 hour	After 2 hours	After 3 hours
XV	5 g.	40·1	40·9	40·1	40·2
XVI	,,	40·2	42·4	41·0	40·5

The change in the alveolar CO_2 is extremely slight, but such as it is it reaches a maximum at the end of the first hour.

In order to see whether the change in the CO_2 really reaches its maximal value in a different time from the change in the percentage saturation at 17 mm. a series of observations of the two were undertaken concurrently in the same experiment.

Effect of Sodium lactate on Percentage Saturation and Alveolar Carbonic Acid.

No. of exp.	Dose of sodium lactate		Before	After 1 hour	After 2 hours	After 3 hours
XVII	5 g.	% sat.	73·1	73·1	83·3	72·2
		Alv. CO_2 mm.	39·5	43·0	42·3	40·1
XVIII	,,	% sat.	74·0	80·5	85·3	74·0
		A.v. CO_2 mm.	40·1	41·9	39·7	39·4

These figures bear out what I found in the earlier observations, namely, that the trifling change in CO_2 pressure is most evident at the end of the first hour and the change in percentage saturation at the end of the second (Fig. 2).

It was a matter of some interest to ascertain whether a similar result would be obtained when sodium bicarbonate was taken (Fig. 3).

Effect of Sodium bicarbonate on Percentage Saturation and Alveolar Carbonic Acid.

No. of exp.	Dose of $NaHCO_3$		Before	After 1 hour	After 2 hours	After 3 hours
XIX	4 g. (3·8 g. equivalent to 5 g. sodium lactate)	% sat.	78·2	83·5	90·3	83·9
		Alv. CO_2 mm.	40·4	40·3	40·9	40·5
XX	,,	% sat.	73·9	81·5	83·9	76·9
		Alv. CO_2 mm.	40·6	40·9	40·7	41·0

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Conclusions.

Two points seem to be established.

(1) Urea, sodium lactate and sodium bicarbonate, in the doses taken, produce little change on the alveolar CO_2.

(2) These substances produce an appreciable change on the affinity of blood for oxygen in the presence of CO_2.

It remains to investigate the question: Do these compounds affect the percentage saturation of the blood with oxygen in the presence of the alveolar pressure of CO_2? So far as sodium lactate in 5 gram doses is concerned the effect does not appear to be appreciable:

Effect of Sodium lactate on Percentage Saturation in the Presence of 27·7 mm. of Oxygen and 40 mm. CO_2.

No. of exp.	Dose	Before	After 2 hours
XXI	5 g. sodium lactate	57·4 (Alv. CO_2=40)	58·5 (Alv. CO_2=39)
XXII	,,	47·1 (Alv. CO_2=40)	45·8 (Alv. CO_2=39)

I am indebted with warmest thanks to Mr Barcroft for his kind guidance in the performance of this investigation.

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XLVII. THE VOLUMETRIC ESTIMATION OF TOTAL SULPHUR AND SULPHATES IN SMALL QUANTITIES OF URINE.

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(*Received November 8th, 1915.*)

Introductory.

A large number of publications have appeared from time to time upon the estimation of sulphates by the benzidine method of Raschig [1903]. The majority, if not all, of these communications have declared that, in general inorganic analysis, the method is one on which the utmost reliance is placed.

The method, which has as its basis the fact that benzidine sulphate is very insoluble in water, depends upon the precipitation of the sulphate present by means of a dilute solution of benzidine hydrochloride. The insoluble benzidine sulphate is then removed by filtration, and can be accurately titrated against standard alkali, as benzidine salts are readily dissociable. The application of the method to the analysis of biological material was not attempted until two papers appeared, almost simultaneously, by Rosenheim and Drummond [1914][1], and Gauvin and Skarzynski [1913].

Both these papers dealt with the analysis of urine by this method. That of Rosenheim and Drummond contained a systematic investigation into the conditions necessary for accurate analyses to be made, and showed that the inorganic and ethereal sulphates in urine could be rapidly and accurately determined by the benzidine method. These authors also suggested that the method could be adapted for the analysis of very small quantities of urine and for the determination of total sulphur.

[1] Rosenheim and Drummond's results were communicated to the Biochemical Society at a meeting held on November 20th, 1913.

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This was partially borne out by the independent findings of Gauvin and Skarzynski, who showed that, by applying the benzidine method to the solution of salts obtained after oxidising the sulphur in urine by various fusion methods, analyses of the total sulphur present might be made.

These two authors also confirmed the results of Rosenheim and Drummond for the analysis of the total and inorganic sulphates in urine, although they did not, apparently, investigate so closely the conditions necessary for accuracy.

Subsequently Raiziss and Dubin [1914] made use of the benzidine method in the determination of total sulphur in small quantities of urine, and in their communication made some criticisms of the titration of the benzidine sulphate with alkali.

These workers stated that the method was not trustworthy for the analysis of small quantities of urine, as they found that the quantity of sulphuric acid liberated from 10 cc. of urine was too small to be accurately titrated with 0·1 N alkali, and further, they were unable to obtain a sharp end-point to the titration, except after repeated additions of alkali and boilings. To surmount these apparent disadvantages, they devised an alternative method of titrating the benzidine sulphate, which was based on an observation that benzidine sulphate may be quantitatively oxidised by means of potassium permanganate.

Their paper only deals with the analysis of the total sulphur in urine by this method, applying the precipitation of the benzidine sulphate to the final solution of salts obtained after oxidising the urine by the method of Benedict [1909], as modified by Denis [1910]; and they claim considerable accuracy for the method even in the analysis of as small a volume as 2 cc. of urine. The present communication records the results of a series of experiments which were carried out to test whether the earlier method might not be used successfully for the micro-estimation of the sulphates in urine and the total sulphur in biological material in general.

Incidentally, the method devised by Raiziss and Dubin was investigated, mainly with the object of determining whether any great advantage lay in the titration of the benzidine sulphate with permanganate, which they advocate, instead of with alkali. Naturally, the much larger titration figure, which is obtained in the permanganate method, is a distinct advantage when small quantities of benzidine sulphate are being dealt with, but I have not been convinced that this is in itself sufficiently important to necessitate an alteration in the method.

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The objections raised by the American workers to the titration of the benzidine sulphate with alkali appear to have little foundation, for, first, I have never yet, in the course of many hundred urinary analyses by this method, encountered an indefinite end-point to a titration; provided that during the filtration strict care is paid to certain details of technique. If the precipitated benzidine sulphate is allowed to suck dry upon the filter, it will form a glistening layer which will not disintegrate upon suspension in water, but will float about in the liquid in the form of small flakes, which are not readily attacked by the alkali during titration. Should the precipitate be once obtained in this form, an end-point by direct titration with alkali is only possible after "repeated boilings and additions of alkali." However, even in this case, a rapid and accurate titration may be made by dissolving the precipitate in an excess of standard alkali by warming, and then titrating that excess with standard acid.

The great importance of the details of technique, which must be observed during filtration, have been emphasised by almost all the investigators who have used the method; hence it is possible that Raiziss and Dubin failed to carry out the filtration in an entirely satisfactory manner.

Their other objection to the titration of the benzidine sulphate with alkali, namely, that 10 cc. of urine yields too small a quantity of sulphuric acid to titrate accurately with standard alkali, receives its answer in the results recorded in the present paper, which show that by the employment of a more dilute alkali, 0·02 N, very accurate analyses of much smaller volumes of urine than 10 cc. may be made.

The method employed by Raiziss and Dubin is as follows. The precipitated benzidine sulphate is filtered off upon an asbestos filter, and is then dissolved in a comparatively large volume of dilute sodium hydroxide, by warming. The solution thus obtained is cooled to room temperature, and, by the addition of a certain volume of concentrated sulphuric acid, is brought to an empirical temperature at which titrations are made with standard potassium permanganate.

In order to standardise the permanganate solution, and obtain its value in terms of sulphur, they carried out estimations upon a pure sulphuric acid solution, and they thus arrived at the factor 0·000099 to represent the value of 1 cc. 0·1 N potassium permanganate in grams of sulphur. Utilising this factor they calculate their results for the analysis of urine.

Upon trying the method, as described by them, I found that in the analysis of pure solutions of sulphates there is no difficulty in the technique. It was

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my experience, however, that the end-point here really does leave much to be desired. This is particularly the case when one is titrating comparatively large quantities of benzidine sulphate.

The titration fluid passes through various shades of orange and yellow, until shortly before the end-point is reached one has an almost colourless solution, and I have often found it most difficult to determine precisely when I have obtained the permanent pink coloration, lasting for twenty seconds, which is advised by the authors.

The empirical temperature of the titration seems an unsatisfactory condition, and, in my experience, unreliable results may occur if one does not always titrate at the same temperature.

TABLE I.

Solution analysed	Cc. analysed	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·1 N $KMnO_4$	% S	Factor (g. of S equivalent to 1 cc. $KMnO_4$)
Solution 1	20	0·2402	—	—	0·164	—
0·1 N H_2SO_4	,,	0·2394	—	—		—
	10	—	9·99	—		—
	,,	—	9·99	—		—
	,,	—	9·99	—	0·1602	—
	,,	—	10·00	—		—
	,,	—	10·02	—		—
	0·5	—	—	8·44	—	
	,,	—	—	8·55	—	
	,,	—	—	8·49	—	0·0000944 g. S
	,,	—	—	8·57	—	
	,,	—	—	8·52	—	
Solution 2	50	0·0918	—	—	0·0249	—
Sodium	,,	0·0907	—	—		—
sulphate	25	—	3·91	—		—
containing	,,	—	3·92	—		—
0·0245 % S	,,	—	3·91	—	0·0250	—
	,,	—	3·93	—		
	,,	—	3·90	—		—
	2	—	—	5·39	—	
	,,	—	—	5·30	—	
	,,	—	—	5·30	—	0·0000939 g. S
	,,	—	—	5·42	—	
	,,	—	—	5·30	—	
Solution 3	20	0·0216	—	—	0·0148	—
Sodium	,,	0·0216	—	—		—
sulphate	100	—	8·88	—		—
containing	,,	—	8·91	—	0·0143	—
0·0145 % S	,,	—	8·92	—		—
	,,	—	8·90	—		—
	5	—	—	7·73	—	
	,,	—	—	7·70	—	
	,,	—	—	7·75	—	0·0000937 g. S.
	,,	—	—	7·72	—	

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As a result of the analysis of many pure solutions of sulphates, I have not obtained a constant value to express the strength of the permanganate solution in terms of sulphur. The factor is an empirical one and considerably influenced by the experimental conditions. As a rule I have obtained somewhat lower figures than the 0·000099 given by Raiziss and Dubin. In the analysis of urine by this procedure, good results can be obtained if the permanganate is standardised against a pure sulphate solution under certain experimental conditions, and the urinary analyses are then conducted under identical conditions. The method, however, is in no way superior to the usual process, in which the benzidine sulphate is titrated directly in aqueous suspension with standard alkali.

Some of the analyses obtained upon pure sulphate solutions may be recorded here (Table I).

Having come to the conclusion that the permanganate titration suggested by Raiziss and Dubin possessed no advantages over the alkali titrations used by earlier authors, it was then necessary to determine first, whether the alkali titration process was adaptable to the estimation of small quantities of sulphates, and if so, to apply it to the estimation of the sulphates in small volumes of urine; and secondly, how best to utilise the process in the estimation of total sulphur.

Experimental.

1. The Estimation of small quantities of Sulphates in pure solution by the Benzidine method.

In the first place, it was found that the precipitation of very small quantities of sulphates from pure solutions as benzidine sulphate was not quantitative unless the reaction of the fluid was carefully adjusted. Rosenheim and Drummond pointed out that an excess of hydrochloric acid prevented the quantitative precipitation of benzidine sulphate, and it was found that only a faint acid reaction must be present during precipitation. If this is so the benzidine sulphate separates quantitatively. The separation of the small precipitates thus obtained was attempted by several means, such as centrifugalisation, but it was finally found that the simple filtration method could not be improved upon. Naturally, the greatest care must be paid to the details of technique during filtration when very small precipitates are dealt with. The details are as follows:

A 5 cm. funnel is fitted into the neck of a 250 cc. filtering flask and carries

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a small perforated porcelain filtering disc of 1 cm. diameter. A suspension of filter paper pulp is poured into the funnel and sucked dry upon the porcelain disc by means of a filter pump. The layer of paper should not be too thick; 1–2 mm. is sufficient to retain benzidine sulphate crystals. The benzidine sulphate precipitate can now be directly poured on to the filter, and filtered by the application of a partial vacuum. Care must be taken to prevent the precipitate at any time sucking dry upon the filter, otherwise, as previously stated, it will be obtained in a flaky condition and will not be attacked by the alkali during the titration [Raschig, 1903; Järvinen, 1913]. The pressure is therefore gently released at the moment before the precipitate sucks dry upon the filter. The vessel in which the precipitation has been carried out is then washed out into the funnel with two washings of about 2–3 cc. of a saturated solution of benzidine sulphate in distilled water, and the washings drawn through the filter by gentle suction, again taking care to prevent the precipitate being sucked dry. It is not advisable, especially when dealing with small precipitates, to wash the precipitate with distilled water, as do Raiziss and Dubin, because of the slight, but appreciable solubility of benzidine sulphate in water.

The precipitate, together with the filter paper and porcelain disc, are transferred by means of a clean glass rod into the vessel in which the precipitation was carried out, and the rod and funnel washed into it with a fine jet of distilled water.

The suspension of benzidine sulphate and filter paper, which, provided care has been taken during filtration, should show no flakes of benzidine sulphate, is then heated to boiling-point and titrated whilst hot with 0·02 N potassium hydroxide. As an indicator a dilute solution of phenolphthalein, or a drop or two of a saturated alcoholic solution of methyl red, can be used. Both indicators yield sharp end-points.

It is always necessary, when titrating benzidine sulphate precipitates with 0·02 N alkali, to carry out a blank estimation upon the reagents for each series of analyses.

Should the benzidine sulphate precipitate be obtained upon the filter in the form of flakes which cannot be titrated with alkali, all that is necessary to obtain an accurate result is to dissolve the precipitate in an excess of standard alkali by warming, and then to titrate the excess of alkali with standard acid, using either of the indicators mentioned above.

The following results, which have been obtained upon a pure solution of sodium sulphate, demonstrate the accuracy of the method.

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TABLE II.

Analysis of a Solution of Sodium sulphate containing 0·0248 % *S.*

Cc. analysed	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% S
50	0·0914	—	—	0·0250
,,	0·0910	—	—	
25	—	3·91	—	
,,	—	3·92	—	0·0251
,,	—	3·91	—	
,,	—	3·93	—	
10	—	—	8·04	
,,	—	—	7·93	
,,	—	—	8·00	0·0253
,,	—	—	7·96	
,,	—	—	7·96	
5	—	—	3·94	
,,	—	—	3·88	
,,	—	—	3·92	0·0249
,,	—	—	3·94	
,,	—	—	3·86	
2	—	—	1·56	
,,	—	—	1·58	
,,	—	—	1·59	0·0252
,,	—	—	1·56	
,,	—	—	1·60	

Having determined definitely that upon pure solutions the method yielded highly accurate results, the application was extended to the micro-analysis of the urinary sulphates.

2. Estimation of small quantities of Urinary Sulphates.

2 *a*. *Inorganic sulphates.* The estimation of the inorganic sulphates in urine presented no difficulty whatever, it being quite sufficient to apply on a smaller scale the method as described by Rosenheim and Drummond for 20 cc. of urine. Accordingly the following technique was adopted and has yielded very accurate figures:

5 cc. of urine are measured into a 50 cc. beaker, acidified with 0·5 cc. of a 1 in 4 solution of hydrochloric acid (1 part conc. HCl to 3 of H_2O), and 20 cc. of the benzidine reagent are then added. After allowing the precipitate to settle for five minutes, it is filtered off upon a small filter-paper pulp filter in exactly the same way as has just been described for the estimation of pure sulphate solutions. The precipitate is transferred to the original beaker, and titrated at boiling-point with 0·02 N potassium hydroxide, using either phenolphthalein or methyl red as an indicator.

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Quantities of urine as small as 2 cc. may be estimated by an exactly similar procedure, using proportional quantities of reagents. Analyses of the inorganic sulphates in two samples of urine are here shown. The control estimations were in all cases made by Folin's gravimetric method [1906].

TABLE III.

Sample	Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% SO_3
Urine 1	20	0·1069	—	—	0·183
	,,	0·1067	—	—	
	20	—	9·10	—	
	,,	—	9·00	—	
	,,	—	9·10	—	0·182
	,,	—	9·15	—	
	,,	—	9·12	—	
	10	—	4·55	—	
	,,	—	4·55	—	0·182
	,,	—	4·55	—	
	,,	—	4·55	—	
	5	—	—	11·55	
	,,	—	—	11·37	
	,,	—	—	11·25	
	,,	—	—	11·45	0·183
	,,	—	—	11·40	
	,,	—	—	11·46	
	,,	—	—	11·42	
Urine 2	20	0·0916	—	—	0·157
	20	—	7·70	—	
	,,	—	7·62	—	0·153
	,,	—	7·66	—	
	,,	—	7·60	—	
	10	—	3·88	—	
	,,	—	3·88	—	0·154
	,,	—	3·80	—	
	,,	—	3·82	—	
	5	—	—	9·76	
	,,	—	—	9·46	
	,,	—	—	9·79	0·154
	,,	—	—	9·56	
	,,	—	—	9·59	
	2	—	—	3·85	
	,,	—	—	3·87	
	,,	—	—	3·96	0·157
	,,	—	—	4·00	
	,,	—	—	4·00	

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2 *b*. *The estimation of total sulphates.* This presented more difficulties than did the determination of the inorganic sulphates, mainly because the most careful regulation of the acidity after hydrolysis had to be made, in order that the precipitation of the benzidine sulphate should be complete.

In the earlier analyses, this was carried out as described by Gauvin and Skarzynski [1913], who, after hydrolysis of the ethereal sulphates in the usual manner, neutralised their solution with alkali and then reacidified it with the correct amount of hydrochloric acid. This procedure yielded fairly good results, but it was found, during some preliminary experiments, that there was no need to go to this trouble, as the ethereal sulphates in urine may be quantitatively hydrolysed by a far smaller proportion of hydrochloric acid than is usually employed.

Equal volumes of a sample of urine were hydrolysed by boiling with varying proportions of 1 in 4 hydrochloric acid, under identical conditions for 30 minutes. The analyses of total sulphates made on each sample, which are given below, demonstrate fully that the ethereal sulphates present in 20 cc. of urine are as completely hydrolysed by boiling for half an hour with 2 cc. of 1 in 4 hydrochloric acid as by boiling under identical experimental conditions with 15 cc. of the acid.

TABLE IV.

Urine hydrolysed for 30 minutes over constant source of heat.

Estimation	Cc. urine	Cc. 1 in 4 HCl	Weight of $BaSO_4$ in g.	% SO_3
Total sulphates	20	15	0·0824	0·142
	20	15	0·0828	
,,	20	10	0·0827	0·142
,,	20	10	0·0829	
,,	20	7	0·0826	0·142
,,	20	7	0·0835	
,,	20	5	0·0830	0·142
,,	20	5	0·0826	
,,	20	3	0·0837	0·143
,,	20	3	0·0835	
,,	20	2	0·0834	0·143
,,	20	2	0·0836	
Inorganic sulphates	20	—	0·0771	0·133
	20	—	0·0778	

This fact at once simplified the estimation of the total sulphates in urine on a small scale, and the method finally adopted as yielding the most reliable results is as follows.

5 cc. of urine are measured into a 25 cc. wide mouthed Erlenmeyer flask, and 0·5 cc. of a 1 in 4 solution of hydrochloric acid is added. A small glass

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funnel inserted in the neck of the Erlenmeyer flask acts as a reflux trap to prevent spurting and undue concentration of the contents. Hydrolysis is then effected by boiling the liquid gently on a sand-bath for 30 to 40 minutes. The flask should be kept under observation during this period, and any appreciable diminution in the volume of the contents of the flask readjusted by the addition of a few drops of distilled water.

At the end of the period of time stated, the flask is removed from the sand-bath and allowed to cool. 20 cc. of the benzidine reagent are added, after the funnel and the neck of the hydrolysis flask have been washed down with a fine jet of distilled water. The precipitate is allowed to settle for five minutes, and is then filtered off and titrated in the manner already described.

The following urinary analysis will serve to show that the analysis of the total sulphates in as small a volume of urine as 2 cc. is rapidly and accurately made by this method.

TABLE V.

Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% SO_3
20	0·0824	—	—	0·142
,,	0·0824	—	—	
10	—	3·56	—	
,,	—	3·57	—	0·142
,,	—	3·54	—	
,,	—	3·58	—	
5	—	—	8·78	
,,	—	—	8·80	0·141
,,	—	—	8·78	
,,	—	—	8·79	
2	—	—	3·50	
,,	—	—	3·49	0·140
,,	—	—	3·46	
,,	—	—	3·47	

2 *c*. *Pathological urines.* Many pathological urines have been analysed by the method, and, in all but a few cases, have yielded highly accurate results. The presence of pigments in some pathological urines may render the hydrolysed urine dark in colour, and the precipitated benzidine sulphate will then often carry down much of the coloration with it. Usually the benzidine sulphate precipitate thus obtained is of a pale mauve colour, and this coloration is apt to interfere somewhat with the end-point of the titration. No means has yet been found of preventing this, but personally I have found little difficulty in deciding the end-point in such cases, if phenolphthalein is used as the indicator, and one has a little experience of the method.

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The presence of albumin at first presented difficulties in the analysis of some urines, but I have found that if the method recently described by Tracey and Welker [1915] for the removal of albumin from urine is used, accurate results may be obtained in these cases.

To determine this, a sample of urine was taken and divided into two portions. To one was added about 0·7 % of albumin in the form of a serum albumin solution, whilst to the other was added an equal volume of distilled water. The urine containing the albumin was then mixed with an equal volume of aluminium cream suspension, prepared as described by Tracey and Welker, and filtered, whilst the other specimen was diluted with an equal volume of distilled water. Upon analysis the two specimens yielded the following figures, which clearly demonstrate that the removal of albumin from urine by means of its absorption with aluminium cream does not affect the sulphate content of the fluid.

TABLE VI.

Urine	Estimation	Cc. taken	Cc. 0·02 N KHO	% SO_3
Treated	Inorganic sulphates	20	8·60	
		,,	8·25	0·067
		,,	8·34	
		10	4·17	
		,,	4·21	0·067
		,,	4·14	
Untreated	Inorganic sulphates	20	8·40	
		,,	8·25	0·067
		,,	8·20	
		10	4·20	
		,,	4·10	0·066
		,,	4·14	
Treated	Total sulphates	20	9·14	
		,,	9·12	0·073
		,,	9·10	
		10	4·55	
		,,	4·51	0·072
		,,	4·51	
Untreated	Total sulphates	20	9·22	
		,,	9·20	0·073
		,,	9·16	
		10	4·61	
		,,	4·51	0·073
		,,	4·50	

Some typical urinary analyses are here given, to show the high degree of accuracy attainable in analyses by this method.

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TABLE VII.

Sample of urine	Estimation	Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% SO_3
Normal	Inorganic sulphates	20	0·0970	—	—	0·166
		10	—	4·16	—	
		,,	—	4·16	—	0·167
		,,	—	4·20	—	
		,,	—	4·17	—	
		5	—	—	10·20	
		,,	—	—	10·25	0·165
		,,	—	—	10·27	
		,,	—	—	10·27	
		2	—	—	4·18	
		,,	—	—	4·17	0·167
		,,	—	—	4·20	
		,,	—	—	4·18	
	Total sulphates	20	0·1062	—	—	0·182
		10	—	4·56	—	
		,,	—	4·56	—	0·182
		,,	—	4·55	—	
		,,	—	4·57	—	
		5	—	—	11·38	
		,,	—	—	11·35	0·181
		,,	—	—	11·33	
		,,	—	—	11·38	
		2	—	—	4·64	
		,,	—	—	4·68	0·187
		,,	—	—	4·70	
		,,	—	—	4·71	
Normal	Inorganic sulphates	10	—	4·61	—	
		,,	—	4·63	—	0·185
		,,	—	4·62	—	
		,,	—	4·66	—	
		5	—	—	11·78	
		,,	—	—	11·58	0·187
		,,	—	—	11·60	
		,,	—	—	11·62	
		2	—	—	4·78	
		,,	—	—	4·78	0·188
		,,	—	—	4·68	
		,,	—	—	4·68	
	Total sulphates	10	—	4·95	—	
		,,	—	4·89	—	0·196
		,,	—	4·88	—	
		,,	—	4·84	—	
		5	—	—	12·46	
		,,	—	—	12·38	0·198
		,,	—	—	12·50	
		,,	—	—	12·38	
		2	—	—	5·11	
		,,	—	—	5·08	0·204
		,,	—	—	5·12	
		,,	—	—	5·08	

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TABLE VII (*continued*).

Sample of urine	Estimation	Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% SO_3
Tubercular	Inorganic sulphates	10	—	—	8·74	
		,,	—	—	8·72	0·070
		,,	—	—	8·70	
		5	—	—	4·36	
		,,	—	—	4·31	0·069
		,,	—	—	4·40	
		,,	—	—	4·34	
	Total sulphates	10	—	—	9·95	
		,,	—	—	9·97	0·080
		,,	—	—	9·89	
		5	—	—	4·99	
		,,	—	—	4·96	0·080
		,,	—	—	4·95	
		,,	—	—	5·00	
Nephritis; albumin removed by aluminium cream	Inorganic sulphates	10	—	4·62	—	
		,,	—	4·61	—	0·195
		,,	—	4·64	—	
		5	—	—	11·51	
		,,	—	—	11·48	0·184
		,,	—	—	11·56	
		,,	—	—	11·49	
		2	—	—	4·60	
		,,	—	—	4·61	0·184
		,,	—	—	4·56	
	Total sulphates	10	—	5·25	—	
		,,	—	5·20	—	0·208
		,,	—	5·25	—	
		,,	—	5·18	—	
		5	—	—	13·00	
		,,	—	—	13·07	0·208
		,,	—	—	13·07	
		,,	—	—	13·01	
		2	—	—	5·20	
		,,	—	—	5·27	0·208
		,,	—	—	5·21	

3. The Determination of Total Sulphur.

Previous workers who have attempted to apply the benzidine method to the estimation of total sulphur have employed various oxidation methods; thus Gauvin and Skarzynski utilised various fusion methods, whereas Raiziss and Dubin employed the much more convenient process of Benedict, as modified by Denis [1910]. The American authors neutralised the final solution of salts obtained by this method, with sodium hydroxide, then

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slightly re-acidified with hydrochloric acid and precipitated the sulphate present as benzidine sulphate in the usual manner.

The technique I have adopted for this determination resembles this method in its main points, and is as follows.

2 cc. of urine are measured into a 6 cm. porcelain evaporating basin, 0·5 cc. of Benedict's reagent is added, and the fluid evaporated gently to dryness upon the water-bath. The ignition of the residue is, however, carried out entirely by the use of a Barthel alcohol burner. This procedure was adopted as a result of some unpublished observations by Mrs M. C. Rosenheim.

TABLE VIII.

Cystine solution taken, cc.	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% S
5	0·0380	—	—	0·055
,,	0·0381	—	—	
10	—	3·25	—	0·052
,,	—	3·25	—	
,,	—	3·28	—	
,,	—	3·20	—	
,,	—	3·20	—	
,,	—	3·23	—	
2	—	—	3·28	0·0518
,,	—	—	3·26	
,,	—	—	3·23	
,,	—	—	3·20	
,,	—	—	3·24	
,,	—	—	3·23	

She informed me that the variable amount of sulphur found by her in the blank estimations which she made on the purest reagents obtainable were traceable to the sulphur-containing impurities present in London coal gas. I have been able to corroborate this, and have found that if one uses reliable reagents, and conducts the ignition solely with the use of a spirit burner, the blank estimations seldom, if ever, contain detectable quantities of sulphur.

When the residue has been completely ignited over the spirit burner, it is allowed to cool, and 1 cc. of 1 in 4 hydrochloric acid is added. The dish is covered with a clock-glass, and is warmed upon the water-bath until the residue is entirely in solution. Should evaporation to dryness occur before this is the case, another 1 cc. of acid should be carefully added and the warming continued. When the whole of the black residue is in solution, the dish is allowed to remain upon the water-bath, the cover is washed into the dish and the contents gently evaporated to dryness. 1 cc. of distilled water is now added and the contents quantitatively transferred

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to a 25 cc. beaker. 10 cc. of the benzidine reagent are then added to precipitate the sulphate present. The subsequent filtration and titration of the precipitate are carried out in the manner already described.

Analyses carried out upon a solution of pure cystine, for which I am indebted to Dr O. Rosenheim, are given in Table VIII, and are sufficient to show that the method is reliable.

0·1960 g. cystine was dissolved in 5 cc. of hydrochloric acid and diluted to 100 cc. with distilled water; the solution contained 0·052 % sulphur.

The results of the application of the method to the analysis of total sulphur in urine are also given.

TABLE IX.

Sample	Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% S
Urine 1	10	0·0481	—	—	0·066
	,,	0·0486	—	—	
	10	—	4·15	—	0·066
	,,	—	4·10	—	
	,,	—	4·17	—	
	,,	—	4·15	—	
	5	—	—	10·14	0·065
	,,	—	—	10·16	
	,,	—	—	10·10	
	,,	—	—	10·18	
	2	—	—	4·10	0·065
	,,	—	—	4·07	
	,,	—	—	4·11	
	,,	—	—	4·12	
Urine 2	10	0·0439	—	—	0·0604
	,,	0·0442	—	—	
	10	—	3·80	—	0·061
	,,	—	3·82	—	
	,,	—	3·80	—	
	,,	—	3·75	—	
	2	—	—	3·78	0·061
	,,	—	—	3·80	
	,,	—	—	3·83	
	,,	—	—	3·78	

In conclusion I wish to tender my sincere thanks to Dr O. Rosenheim of King's College, for many helpful criticisms and suggestions during the course of this work.

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Summary.

1. The benzidine method of Rosenheim and Drummond for the estimation of urinary sulphates can be carried out upon as little as 2 cc. of urine, by titrating the precipitated benzidine sulphate with 0·02 N alkali.

2. The method is also applicable to the estimation of the total sulphur in small quantities of biological material, by applying the precipitation of the benzidine sulphate to the solution of salts obtained after oxidation of the material by Benedict's method.

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XLVIII. A NEW METHOD FOR THE PREPARATION OF THE PLANT GLOBULINS.

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The plant globulins have been hitherto prepared by the extraction of the material with warm saline solutions. For this purpose sodium chloride has been most frequently employed, and relatively large volumes of solution have been necessary to obtain a satisfactory yield. In the ordinary process, furthermore, several tedious filtrations must be performed, the technique of which has been described in detail by Osborne [see, especially, Osborne, 1909].

It has been shown by Schryver [1910] that the globulins are much more soluble in solutions of those salts which lower the surface tension of water than they are in sodium chloride solution, and it was thought that by the use of these the bulk of material employed in the extraction of the globulins might be very considerably reduced and the troublesome filtration processes to a large extent avoided. At the suggestion of Dr Schryver, I have endeavoured to take advantage of the properties of certain salt solutions already referred to, and have succeeded in elaborating a process by means of which relatively large amounts of plant globulins can be prepared by processes, the chief of which can be completed within the course of a single day. The method is illustrated by the following examples.

Preparation of edestin from hemp-seed.

The hemp-seed is first ground and extracted by light petroleum. It is then filtered off on to a large Buchner-funnel, washed on the funnel with more petroleum and air-dried. The husks are then removed by sifting through coarse muslin.

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500 g. of the material prepared in this way are then mixed with 500 cc. of semi-normal solution of sodium benzoate (about 7 % solution). After standing for about half an hour, so much torn blotting paper is pressed into the mass as to make it appear almost dry. The whole is then wrapped round with filter-paper and placed in a Buchner press and submitted to high pressure. About 400 cc. of liquid can be expressed. The residual mass is extracted again with about 500 cc. of the benzoate solution, and after standing for a short time, more blotting paper is added (relatively, only a small amount is necessary) and the whole is again pressed in the Buchner press, a further quantity of extract amounting to about 500 cc. being thereby obtained. The amount of edestin remaining in the residue is so small that a third extraction is almost superfluous.

The combined extracts are then thrown into about 10 litres of cold water and the whole is allowed to stand over night in a cool place. The edestin separates at the bottom of the vessel; the clear liquid is decanted off, and the creamy paste at the bottom is centrifuged. The edestin separates as a compact powder, and is then washed on the centrifuge once or twice with water, once with 95 % alcohol, once with absolute alcohol, and twice with dry ether. It is then rapidly transferred to a vacuum desiccator. A product is thereby obtained which is almost completely soluble in 10 % sodium chloride solution from which it can be obtained in a crystalline form in the usual manner. Any small impurity which is insoluble in the sodium chloride solution can be separated by centrifugalisation. 130 g. of the crude edestin (vacuum-dried) were obtained from 450 g. of the sifted meal. Semi-normal sodium salicylate solution was also tried instead of the benzoate. A violet coloured product is in this case generally obtained, owing to the action of the salicylate on the metal of the press. This colour is retained in an obstinate manner by the globulin even after recrystallisation.

Preparation of excelsin from Brazil-nuts.

The shelled nuts were passed through a hand mincing machine, and the fat was removed by extraction with light petroleum. 250 g. of fat-free powder were extracted with 250 cc. of semi-normal solution of sodium benzoate, the mass was then mixed with blotting paper, and the liquid expressed in the same manner as that described in the case of edestin. About 145 cc. of extract were obtained. The residue was then extracted twice more, each time with 250 cc., by benzoate solution (with addition of the blotting paper etc.), each extraction yielding after pressure about 250 cc. of

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liquid. The combined extracts were then thrown into 10 litres of water, and the excelsin was separated and treated by the method described in the case of edestin (centrifugalisation, washing, etc.). A yield of about 20 % of the sifted meal was obtained. In another experiment the crude excelsin was dissolved when still moist after washing with water on the centrifuge, in a 6 % solution of ammonium sulphate, and this solution was then centrifuged for about 10 minutes to get rid of small amounts of insoluble matter. After 3 days' dialysis, excelsin separated from the solution, and was centrifuged off, and washed successively with 95 % alcohol, absolute alcohol, and ether, and then vacuum dried. A perfectly white powder was obtained. Yield from 250 g. of meal about 30 g.

By a similar process, a mixture of legumin and vicillin was obtained from horse-beans, and these were separated by the method described by Osborne.

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XLIX. THROMBIN AND CALCIUM CHLORIDE IN RELATION TO COAGULATION.

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(*Received September 24th, 1915.*)

The investigation which forms the subject of this article was undertaken with the object of studying more fully than has hitherto been done the quantitative relation between the time of coagulation of fibrinogen, on the one hand, and the amount of thrombokinase taking part in the process of coagulation, in presence of calcium chloride, on the other hand.

This aspect of blood coagulation has received the attention of several investigators during the last two decades. Thus E. Fuld [1902], using the plasma of geese and turkeys as source of fibrinogen, and as ferment solution the freshly prepared extract, in 0·8 % NaCl solution, of muscle, found that the equation

$$\log \frac{x}{x_0} = 0{\cdot}585 \log \frac{y}{y_0}$$

is valid when $y =$ the amount of ferment corresponding to coagulation time x and y_0 similarly corresponds to x_0.

C. J. Martin [1905], employing oxalated plasma of the sheep and dog, found that the coagulation time multiplied by the quantity of ferment (snake venom) was constant, but this law failed when a relatively large amount of venom was added, the observed times being then longer than the calculated periods. L. J. Rettger [1909] observed that the product of the coagulation time and the amount of thrombin employed was constant if the amount of fibrinogen used was fixed, but that if excess of thrombin was used a maximum amount was reached beyond which further increase no longer diminished the time of coagulation; the latter represented the time at which the contents

[1] Towards the expenses of this research a grant was made by the British Medical Association.

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of the tube were sufficiently coagulated for the tube to be inverted, without the contents being spilt.

It early became obvious in the course of my investigation that the fowl's plasma employed in the experiments contained some thrombokinase, for although, when oxalated, it remained free from coagulation (a varying amount of white precipitate made its appearance on keeping) during the period it was kept under observation (four weeks at 32° C.), showing that it did not contain thrombin in appreciable quantity, nevertheless on the addition of calcium chloride coagulation always occurred after a shorter or longer period of time. It was also clear that the amount of thrombokinase present in different samples of fowl's plasma exhibited great variation. Thus coagulation at 35° C. sometimes occurred at the end of thirty minutes after the addition of calcium chloride, while when separation of plasma had been more successful its occurrence under the same conditions was not observed until the expiration of several hours. This separation was accomplished by collecting blood from the femoral artery in paraffined tubes by a paraffined cannula and obtaining plasma therefrom by centrifugalisation. Similar variations in respect of spontaneous coagulability were observed in fibrinogen solutions prepared from such samples of plasma by dilution with twenty parts of distilled water through which a stream of CO_2 had been passed. The resulting precipitate of fibrinogen was collected by centrifugalisation, dissolved in 0·85 % NaCl solution and again centrifugalised, the bulk of the resulting solution being made equal to that of the plasma, from which the fibrinogen was obtained. It was therefore clear that in estimating the coagulative effect of a given amount of thrombokinase it would be necessary to ascertain, if possible, the quantity already present in the fibrinogen solution or plasma employed. The mode in which this is effected will now be described.

In Table I a series of experiments is shown in which varying amounts of a solution of thrombokinase prepared from the testis of the rabbit were added to 0·325 cc. of fibrinogen solution in the presence of 0·05 cc. of N/10 $CaCl_2$ solution, the total volume of fluid being in each case made up to 0·5 cc. and the time of coagulation noted. The solution of thrombokinase was prepared by grinding (in a mortar) the body of the testis with sand in presence of distilled water, the resulting emulsion being made up to the original volume of the testis or to any desired multiple of the same. The extract so obtained was centrifugalised in order to remove all coarse suspended particles.

In attempting to discover if any relation exists between the time of coagulation, x (i.e. the period required for *complete* separation of fibrin),

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and the amount of thrombokinase, y, added it will be observed that the product xy does not vary greatly for the last four experiments. If this relation is assumed to hold good throughout the series, then the amount of thrombokinase present in the 0·325 cc. of fibrinogen solution employed in each experiment can be shown by ordinary algebraical methods, as will be indicated for the experiments given on p. 517, to be equivalent to about 0·0068 cc. of the thrombokinase solution employed; the quantities given in the third column of Table I must therefore be increased by this amount in order that the total quantity of thrombokinase present in each experiment

TABLE I.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen solutions in presence of $CaCl_2$, a solution of NaCl (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	Fibrinogen solution (cc.)	Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	N/10 $CaCl_2$ solution (cc.)	Time of coagulation (35°) (minutes)
1	0·325	—	0·050	408
2	,,	0·097	,,	88
3	,	0·292	,,	30
4	,,	0·877	,,	11
5	,,	2·633	,,	3

* In Experiments 3, 4 and 5 more concentrated solutions of thrombokinase were employed, in order that the total volume of fluid in each tube might not exceed 0·5 cc.

may be exhibited. This correction is made in Table II and the corrected product xy determined. For this the value 9·22 was ultimately selected and it then became possible to recalculate the times of coagulation corresponding to different amounts of thrombokinase from the equation

$$xy = \text{const.} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1),$$

the value of the constant being taken at 9·22. This has been done in the last column of Table II and it will be seen that the observed and calculated coagulation times, except in the case of the first experiment, are in good accord; the calculated curve (the one farthest from the axes of coordinates) is given at A, Fig. 1. The variation observed in the first experiment will come under consideration later, when some of the factors concerned in producing such variations are taken into account.

A further series of experiments was performed, using 0·160 cc. of the same fibrinogen solution, instead of 0·325 cc. These experiments are recorded in

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Table III, the product xy being given in the fifth column, after correction for the amount of thrombokinase present in the smaller amount of fibrinogen solution employed, the latter being equivalent to about 0·00335 cc. of the thrombokinase solution used. Here again the product xy does not differ very greatly in the different experiments. The value selected to represent this product is slightly greater than the mean of the observed values. By the aid of this constant the values of x corresponding to varying amounts of thrombokinase (y) are calculated in the last column of the table (curve B, Fig. 1).

TABLE II.

The data in the second and third columns are taken from Table I.

No. of experiment	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x, corresponding to $y_{corrected}$ (minutes)
1	—	408	0·0068	2·77	9·22	1355
2	0·097	88	0·1038	9·23	,,	89
3	0·292	30	0·2988	8·96	,,	31
4	0·877	11	0·8858	9·74	,,	10·5
5	2·633	3	2·6398	7·92	,,	3·5
				mean 7·92		

TABLE III.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·160 *cc.*) *solutions in presence of* $CaCl_2$ (0·050 *cc. of* $N/10$ *solution*), *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x, corresponding to $y_{corrected}$ (minutes)
6	—	1050	0·00335	3·52	4·54	1355
7	0·048	88	0·05135	4·52	,,	89
8	0·144	30	0·14735	4·42	,,	31
9	0·432	11	0·43535	4·79	,,	10·5
10	1·296	3	1·29935	3·90	,,	3·5
				mean 4·23		

* In Experiments 9 and 10 more concentrated solutions of thrombokinase were employed, in order that the total volume of fluid in each tube might not exceed 0·5 cc.

Yet another series of experiments was made, a still smaller quantity of the fibrinogen solution, namely 0·050 cc., being used for each experiment (Table IV). As before a correction is made for the amount of thrombokinase

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which is present in the fibrinogen solution employed. The resulting values of xy are given in the fifth column of the table. The values of x in the last column are calculated from the constant 1·42, which is somewhat higher than the mean observed value of xy. With the aid of this constant the corresponding curve C shown in Fig. 1 is constructed. It will be noted that

TABLE IV.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·050 *cc.*) *solutions in presence of* $CaCl_2$ (0·050 *cc. of* $N/10$ *solution*), *a solution of* $NaCl$ (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x, corresponding to $y_{corrected}$ (minutes)
11	—	1050	0·00105	1·10	1·42	1355
12	0·015	41	0·01605	0·66	,,	89
13	0·045	22	0·04605	1·01	,,	31
14	0·135	11	0·13605	1·50	,,	10·5
15	0·410	3	0·41105	1·23	,,	3·5
				mean 1·10		

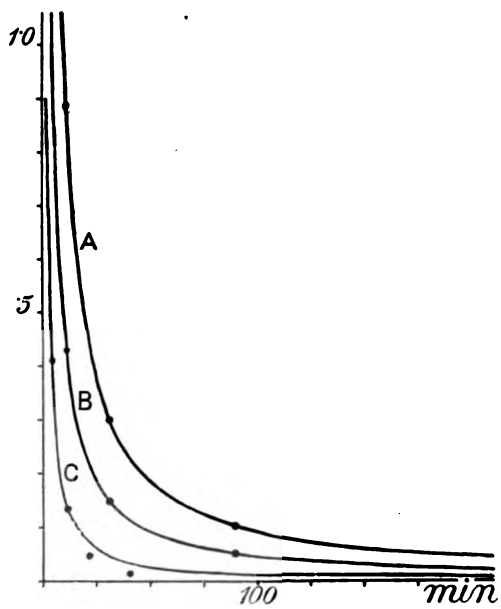

Fig. 1. Curves for the experiments given in Tables I–IV. Abscissae, time in minutes; ordinates, cc. of thrombokinase solution. C corresponds to experiments in which 0·05 cc. of fibrinogen solution was employed; B to 0·160 cc. and A to 0·325 cc.

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in Exps. 11, 12 and 13 the observed are lower than the calculated values; the significance of these variations will be considered later.

All the above series of experiments were made in immediate succession, the same solutions of fibrinogen and thrombokinase being used throughout.

When the mean observed values of $x \times y$ (the latter being corrected for the amount of thrombokinase present in the fibrinogen solution used) were compared, it was found that they were approximately in the ratio of the amounts of fibrinogen solution employed, namely 0·325 cc., 0·160 cc. and 0·050 cc. The values of xy chosen in the above tables are accurately in this ratio and, as the values of x calculated therefrom are in fair agreement with the observed values, it follows that the relation between these quantities is very closely expressed by the equation

$$xy = nz \quad \text{.....(2)},$$

where x represents the time of coagulation, y and z showing the respective amounts of the solutions of thrombokinase and of fibrinogen present in the total volume of 0·5 cc. of fluid employed in each experiment, and n being a constant. To each tube 0·05 cc. of N/10 $CaCl_2$ solution was added; the temperature of experiment was 35°; the value of n is 28·4.

TABLE V.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·050 *cc.*) *solutions in presence of* $CaCl_2$, *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	Fibrinogen solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	N/10 $CaCl_2$ solution (cc.)	x Time of coagulation (35°) (minutes)
11	0·050	—	0·050	46
12	,,	0·040	,,	33
13	,,	0·100	,,	25
14	,,	0·300	,,	13
15	,,	1·000	,,	8

* In Exp. 15 a more concentrated solution of thrombokinase was employed, in order that the total volume of fluid in each tube might not exceed 0·5 cc.

In the above experiments a solution of fowl's fibrinogen was employed, which contained exceedingly little thrombokinase. In the experiments recorded in Table V a relatively large amount of thrombokinase was already present in the fibrinogen solution used, coagulation (at 35°) occurring, after

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the addition of calcium chloride alone, within a period of one hour. In these experiments if y (amount of thrombokinase added) is uncorrected, the product xy increases with each member of the series. The amount, e, of thrombokinase originally present in the quantity (0·050 cc.) of fibrinogen solution introduced into each tube (the total volume of fluid present being as before in each case 0·5 cc.) is determined, in terms of the equivalent amount of the thrombokinase solution employed, from the data given by the first two experiments, by putting the product xe in the former equal to the product $x'(y' + e)$ in the latter. Thus

$$xe = x'(y' + e) \quad \text{.....(3)},$$

whence

$$e = \frac{x'y'}{x - x'} \quad \text{.....(4)}.$$

In this way the following series of values of e is obtained:

Exps.	11 and	12	(Table V)	$e = 0{\cdot}10$,
,,	,,	13	,,	$e = 0{\cdot}12$,
,,	,,	14	,,	$e = 0{\cdot}12$,.
,,	,,	15	,,	$e = 0{\cdot}19$,
				mean 0·13.

The value of e finally chosen was 0·136.

If now to the amounts of thrombokinase introduced into each tube the equivalent (in terms of the thrombokinase solution employed) amount of thrombokinase originally present in the quantity (0·050 cc.) of fibrinogen

TABLE VI.

Recalculation of values of y (thrombokinase) and x (time of coagulation) from Table V.

No. of experiment	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Corresponding value of $x_{calculated}$ (minutes)	x_{found} (minutes)
11	0·136	6·25	6·0	44	46
12	0·176	5·80	,,	34	33
13	0·236	5·90	,,	25	25
14	0·436	5·66	,,	14	13
15	1·136	9·09	,,	5	8
		mean 6·54			

solution used is added, the corrected values of y are obtained and from these the true values of the product xy are found. These are set forth in Table VI. The observed values of x, corresponding to the corrected values of y, are shown in Fig. 2, curve A being calculated for $xy = 6$, this being the value

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ultimately selected. It will be noted that the observed and calculated values agree fairly closely. This series of experiments illustrates how a relatively heavily contaminated solution of fibrinogen—and the same applies to the plasma from which it is obtained—may be employed for the purpose of investigation, provided the amount of admixture of thrombokinase is determined. The disadvantage of such solutions, however, lies in the circumstance that the range of investigation is limited because the time of coagulation for very small concentrations of thrombokinase, corresponding to the lower

TABLE VII.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·160 *cc. for Exps.* 6 *to* 10; 0·325 *cc. for Exps.* 1 *to* 5) *solutions in presence of* $CaCl_2$ (0·050 *cc. of* $N/10$ *solution*), *a solution of* $NaCl$ (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment. These experiments complete the series, of which those recorded in Table V form a part.*

No. of experiment	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x, corresponding to $y_{corrected}$ (minutes)
1	—	52	0·884	45·90	39·0	44
2	0·030	40	0·914	36·56	,,	43
3	0·100	31	0·984	30·52	,,	40
4	0·300	28	1·184	23·14	,,	33
5	1·000	20	1·884	37·68	,,	21
				mean 34·76		
6	—	54	0·436	23·52	19·2	44
7	0·035	38	0·471	17·90	,,	41
8	0·100	30	0·536	16·08	,,	36
9	0·300	27	0·736	19·87	,,	26
10	1·000	14	1·436	20·08	,,	13
				mean 19·49		

* In Exps. 4, 5, 9 and 10 more concentrated solutions of thrombokinase were employed.

parts of the curves shown in the figures (e.g. to the right of the vertical lines at 44 and 27 in Figs. 2 and 3 respectively on the axis of abscissae), cannot be determined, since these represent amounts of thrombokinase which are less than that already present in the plasma or fibrinogen solution employed.

For the sake of comparison with Table VI the remaining experiments of the group from which Table V is constructed are given in Table VII, the corresponding curves (*B* and *C*) being shown in Fig. 2. As in the preceding series the observed values of x do not differ greatly from those

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calculated with the aid of the values of xy chosen (39·0, 19·2 and 6·0), which it will be noted are proportional to the amounts of fibrinogen solution (0·325 cc., 0·160 cc. and 0·050 cc. respectively) employed; thus conforming to equation (2).

The enquiry now presents itself, Does the time of coagulation represent the time required to convert prothrombin into thrombin or does it correspond to the period during which thrombin acts upon fibrinogen, giving rise thereby to the appearance of fibrin or does each of these processes occupy an

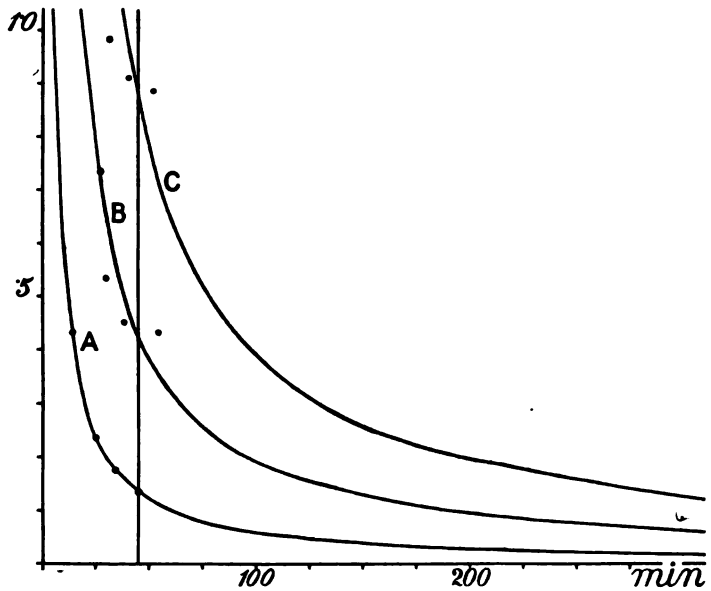

Fig. 2. Curves for the experiments given in Tables V–VII. Abscissae, time in minutes; ordinates, cc. of thrombokinase solution. The vertical line at 44 on the axis of abscissae represents the outer limit of investigation of the curves. Curve *A* corresponds to experiments in which 0·050 cc. of fibrinogen solution was employed; *B* to 0·160 cc. and *C* to 0·325 cc.

appreciable fraction of the coagulation time? To determine this point thrombin in the form of venom from *Echis carinatus* [Barratt 1913, 1] was added to fibrinogen in varying amounts, and the period of coagulation (at 35°) noted. These experiments, which are given in Table VIII, fall into two groups: in the first (Exps. 1 to 14) plasma to which calcium chloride had been added, as in the preceding series, was employed; in the second group (Exps. 15 to 19) oxalated plasma was used. It will be observed that the course of the first group is similar to that of the experiments already recorded, in which thrombokinase was used and, as in the latter, the times

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of coagulation have been calculated on the assumption that the product xy is constant for each of the three amounts of fibrinogen employed, the observed and calculated values being in fair agreement, except for the control experiments 1, 6 and 11, in which the observed periods were longer than the

TABLE VIII.

Determination of the coagulation time of a series of mixtures of venom of Echis carinatus and calcified fowl's plasma (0·325 *cc. for Exps.* 1 *to* 5; 0·160 *cc. for Exps.* 6 *to* 10 *and* 0·050 *cc. for Exps.* 11 *to* 14, *in presence of* 0·050 *cc. of* $N/10$ $CaCl_2$ *solution*) *or oxalated fowl's plasma* (0·325 *cc. for Exps.* 15 *to* 19), *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	y Venom solution, 1 in 300,000* (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x (minutes)	Plasma (cc.)
1	—	52	0·11	5·7	3·00	27	0·325 calcified
2	0·030	20	0·14	2·8	,,	21	,, ,,
3	0·100	14	0·21	3·2	,,	14	,, ,,
4	0·300	4	0·41	1·6	,,	7	,, ,,
5	1·000	2	1·11	2·2	,,	3	,, ,,
6	—	54	0·054	2·9	1·476	27	0·160 ,,
7	0·035	14	0·089	1·2	,,	17	,, ,,
8	0·100	10	0·154	1·5	,,	10	,, ,,
9	0·300	4	0·354	1·4	,,	4	,, ,,
10	1·000	2	0·737†	1·5	,,	2	,, ,,
11	—	44	0·0169	0·74	0·461	27	0·050 ,,
12	0·040	10	0·0469	0·47	,,	10	,, ,,
13	0·100	4	0·1169	0·47	,,	4	,, ,,
14	0·300	2	0·2310†	0·46	,,	2	,, ,,
15‡	—	∞	—	—	3·44	∞	0·325 oxalated
16	0·030	129	—	3·87	,,	115	,, ,,
17	0·100	33	—	3·30	,,	34	,, ,,
18	0·300	9	—	2·70	,,	11	,, ,,
19	0·590	6	—	3·54	,,	6	,, ,,
				mean 3·97			

* In Exps. 4, 5, 9, 10, 14, 18 and 19 more concentrated solutions of venom were employed.
† Effective values of y (cp. p. 526).
‡ In Exps. 15 to 19 the plasma employed was not the same as that used for Exps. 1 to 14.

calculated. In control experiments of this type irregular variation from the calculated periods is met with more frequently than in experiments in which thrombokinase or thrombin has been added. If the experiments are repeated such differences do not appear to be constant, as may be seen by comparing the corresponding experiments in the preceding tables. The degree of

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contamination of the plasma used can be determined as in the previous experiments, except that the estimation is now made in terms of thrombin instead of thrombokinase. Finally it will be noted that the values of xy selected (3·00, 1·476 and 0·461 respectively) are in the same ratio as the amounts of fibrinogen solution added (0·325 cc. in Exps. 1 to 5; 0·160 cc. in Exps. 6 to 10; 0·050 cc. in Exps. 11 to 14), equation (2) being valid, within the limits of experimental error, for these experiments with thrombin. In the second series of experiments shown in Table VIII (Exps. 15 to 19), in which the action of thrombin takes place in presence of potassium oxalate (a solution of fibrinogen different from that employed in the preceding experiments being used), the product xy is, as in the previous experiments, constant and the calculated values of x exhibit a good agreement with the observed values; in these experiments no correction for contamination of plasma is required, the plasma remaining uncoagulated when kept in presence of potassium oxalate. The observed coagulation times are reproduced in Fig. 3; the curves are calculated, *A* representing Exps. 1–5; *B* 6–10 and *C* 11–14.

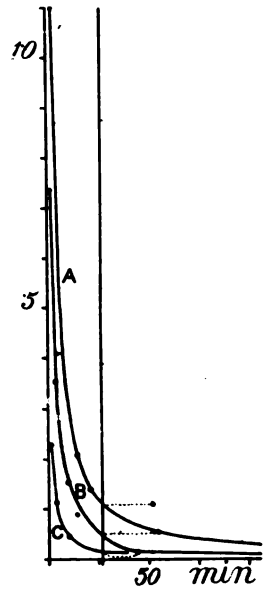

Fig. 3. Curves for the experiments given in Table VIII. Abscissae, time in minutes; ordinates, cc. of 1 in 300,000 venom solution. The vertical line at 27 on the axis of abscissae represents the outer limit of investigation of the curves. Curve *C* corresponds to experiments in which 0·050 cc. of fibrinogen solution was employed, curve *B* to 0·160 cc. and curve *A* to 0·325 cc.

The above experiments, which, like the preceding experiments made with thrombokinase, take place according to equation (2), indicate that the time of coagulation is essentially the period required for the conversion of fibrinogen into fibrin.

Since the experiments with thrombin in Table VIII indicate therefore that the influence of thrombin (y) upon the coagulation time (x) in presence of a given amount of fibrinogen (z) takes place according to equation (2)

$$xy = nz$$

(n in this particular series of experiments being 10·6), and since, in addition, this equation also expresses the action of thrombokinase in causing coagulation

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(Tables I to VII), it follows that thrombokinase and thrombin are interchangeable terms, in respect of fibrin production, in other words that a given amount of thrombokinase, in presence of calcium chloride, will produce a fixed amount of thrombin, whatever may be the concentration of prothrombin, provided of course that the latter is present in excess. As soon as this amount of thrombin has been produced the thrombokinase added has either disappeared or has become so far modified as to be incapable of further action in the direction of giving rise to the production of thrombin in presence of prothrombin and calcium chloride. In no other way can the similarity of the results obtained with thrombin (Table VIII) to those obtained with thrombokinase (Tables I to IV and V to VII) be explained.

The validity of equation (2) may be tested in a manner which in some respects offers an advantage as regards the ease with which simultaneous observations may be made. It is obvious that if, in the equation

$$xy = nz,$$

y is made proportional to z, i.e.

$$y = dz,$$

where d is a constant, then the equation becomes

$$xdz = nz,$$

or

$$x = \frac{n}{d} = \text{const.} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5),$$

that is to say the coagulation time is independent of the concentration of fibrinogen present in solution. This condition is realised if thrombokinase is first added to fibrinogen solution and the mixture thus obtained introduced in varying amounts into tubes, each containing the same quantity of decinormal calcium chloride solution, the total volume of fluid in each case being made up to 0·5 cc. This was in fact done in the series of experiments recorded in Tables I to IV. Thus in the three experiments numbered 2, 7 and 12 a suitable admixture of thrombokinase and fibrinogen solutions was first made and from this amounts of 0·422 cc., 0·208 cc. and 0·065 cc. (containing respectively 0·325 cc., 0·160 cc. and 0·050 cc. of fibrinogen solution) were transferred to tubes into which the requisite amounts of N/10 $CaCl_2$ solution and 0·85 % NaCl solution had already been introduced; and so on with the remaining tubes with the exception of those numbered 1, 6 and 11, to which no thrombokinase was added.

It will be observed that corresponding tubes exhibit equi-coagulative periods, except in Exps. 12 and 13, Table IV; some of the causes of such variations are considered on p. 540.

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The influence of calcium chloride upon the action of thrombokinase and thrombin in effecting coagulation of a fibrinogen solution.

The constant n in equation (2) (p. 516) is, in the mathematical sense, a function of all those potential variables which for these experiments are kept constant. Of the latter two are more immediately important, namely the concentration of $CaCl_2$ and the temperature, the former of which alone will be considered here.

To this end a series of forty experiments with thrombokinase, similar in type to those shown in Tables I and II, was carried out in the numerical order given in Tables IX and X. These experiments fall into two groups: in the first (Table IX) 0·325 cc. of fibrinogen solution was employed in each experiment; in the second (Table X) 0·160 cc. of fibrinogen solution was used.

TABLE IX.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·325 *cc.*) *solutions in presence of* $CaCl_2$, *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	N/10 $CaCl_2$ solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	Value of xy selected	$x_{calculated}$ (minutes)
1	0·075	0·1	44	0·148	7·98	54·0
3	,,	0·2	31	0·248	,,	32·2
5	,,	0·3	23	0·348	,,	22·9
7	,,	0·6	13	0·648	,,	12·2
9	,,	1·0	10	1·048 [0·897]	,,	7·6 [8·9]
11	0·125	0·1	50	0·148	9·42	63·6
13	,,	0·2	39	0·248	,,	38·0
15	,,	0·3	23	0·348	,,	27·1
17	,,	0·6	13	0·648	,,	14·5
19	,,	1·0	10	1·048 [0·897]	,,	9·0 [10·5]
21	0·5	0·1	300	0·148	42·8	289·5
23	,,	0·2	129	0·248	,,	173·0
25	,,	0·3	100	0·348	,,	123·2
27	,,	0·6	59	0·648	,,	66·0
29	,,	1·0	46	1·048 [0·897]	,,	40·8 [47·8]
31	1·5	0·1	>6500	0·148	327·5	2218
33	,,	0·2	>5000	0·248	,,	1323
35	,,	0·3	>4000	0·348	,,	944
37	,,	0·6	1200	0·648	,,	505
39	,,	1·0	780	1·048 [0·897]	,,	313 [365]

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In these two tables the experiments are seen to be arranged in four groups, each of five experiments, the amounts of decinormal $CaCl_2$ solution employed in each group being respectively 0·075 cc., 0·125 cc., 0·5 cc. and 1·5 cc. The experiments in each individual group are arranged, as in the preceding tables, in the order of the amount of thrombokinase introduced into each tube. As in the preceding experiments each tube contained 0·5 cc. of fluid. When the amount of thrombokinase or calcium chloride solution was so great that this amount would have been exceeded, more concentrated solutions were employed, but in the tables, for the sake of uniformity and consequent ease of calculation the amount used is always referred to the same standard of concentration.

As before the first step in dealing with these experiments was to determine, by means of the method given on p. 517, the amount of thrombokinase contained in the fibrinogen solution used and, at the same time, to ascertain if for each group values of xy could be obtained which would satisfy equation (1) (p. 513), namely

$$xy = \text{const.}$$

The amount of thrombokinase present in 0·325 cc. and 0·160 cc. of the fibrinogen solution employed was estimated to be equal to 0·048 cc. and 0·024 cc. respectively of the thrombokinase solution used. When these quantities were added to the thrombokinase employed, the total amount of thrombokinase taking part in each experiment became known and it was possible to select values representing the product of x (coagulation time) and y (amount of thrombokinase present) for each group. The values of xy so chosen yield, it will be seen, calculated values of x differing (except in cases to which reference will shortly be made) from the observed values only within the limits of experimental error.

It will further be seen that the values of xy (7·98 and 3·61 respectively) selected for the first group in Tables IX and X, in which the amount of $CaCl_2$ solution employed is lowest, are approximately in the ratio of the amounts of fibrinogen solution added (0·325 cc. and 0·160 cc. respectively), but that with increasing amounts of $CaCl_2$ a departure from this ratio is observed.

It has already been pointed out that certain calculated values of x differ from those actually observed, to an extent which lies beyond the range of experimental error. These fall into two groups: (1) those in which a very large amount of thrombokinase has been employed (Exps. 9, 19, 29 and 39, Table IX, and 8, 10, 18, 20, 28, 30, 38 and 40, Table X); and (2) those in

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which a very large amount of $CaCl_2$ has been used (Exps. 31 to 40). The latter group will be dealt with later but the former may now conveniently be considered. For a long time this class of variations from the calculated value of the coagulation time (x) presented a difficulty, until it was observed that the coagulation time became stationary as soon as the amount of

TABLE X.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·160 *cc.*) *solutions in presence of* $CaCl_2$, *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	N/10 $CaCl_2$ solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	Value of xy selected	$x_{calculated}$ (minutes)
2	0·075	0·1	30	0·124	3·61	29·1
4	,,	0·2	20	0·224	,,	16·1
6	,,	0·3	13	0·324	,,	11·1
8	,,	0·6	10	0·624 [0·442]	,,	5·8 [8·2]
10	,,	1·0	10	1·024 [0·442]	,,	3·5 [8·2]
12	0·125	0·1	39	0·124	3·73	30·1
14	,,	0·2	17	0·224	,,	16·7
16	,,	0·3	11	0·324	,,	11·5
18	,,	0·6	7	0·624 [0·442]	,,	6·0 [8·5]
20	,,	1·0	7	1·024 [0·442]	,,	3·6 [8·5]
22	0·5	0·1	56	0·124	6·59	53·2
24	,,	0·2	26	0·224	,,	29·4
26	,,	0·3	20	0·324	,,	20·4
28	,,	0·6	15	0·624 [0·442]	,,	10·6 [14·9]
30	,,	1·0	12	1·024 [0·442]	,,	6·4 [14·9]
32	1·5	0·1	>6500	0·124	31·03	250·0
34	,,	0·2	1440	0·224	,,	138·7
36	,,	0·3	780	0·324	,,	95·7
38	,,	0·6	780	0·624 [0·442]	,,	49·7 [70·2]
40	,,	1·0	780	1·024 [0·442]	,,	30·3 [70·2]

thrombokinase (y) reached a certain value, beyond which further increase caused no appreciable shortening of the time of coagulation. Whatever may be the mode, physical or physico-chemical, in which this event takes place, its occurrence may be conveniently expressed by saying that in practice the effective maximum value of thrombokinase is fixed and cannot be exceeded. This value was determined approximately by substituting varying lower values of y for the highest amounts actually employed and selecting those

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which gave the best concordance between the calculated and observed values of x[1]. Since the maximum effective values of y which were at first selected for the two series of experiments (Tables IX and X) were found to be approximately in the ratio of the amounts of fibrinogen solution employed, it was assumed that this relation actually existed and on this assumption the values finally adopted were chosen (0·897 cc. of thrombokinase solution when

TABLE XI.

The experiments recorded in Table IX, rearranged in a form suitable for calculating the values of a and b in the equation $w^2 = ax - b$. The observed coagulation times are given within brackets in the fourth column.

No. of experiment	w N/10 $CaCl_2$ solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	$x_{calculated}$ Time of coagulation (minutes)		$a = \frac{w_1^2 - w^2}{x_1 - x}$	$b = ax - w^2$
9	0·075	0·897	[10]	8·9	0·0062	0·0504
19	0·125	,,	[10]	10·5	0·0063	,,
29	0·5	,,	[46]	47·8	,,	,,
39	1·5	,,	[780]	365·0	,,	,,
7	0·075	0·648	[13]	12·2	0·00455	,,
17	0·125	,,	[13]	14·5	,,	,,
27	0·5	,,	[59]	66·0	,,	,,
37	1·5	,,	[1200]	505·0	,,	,,
5	0·075	0·348	[23]	22·9	0·00246	,,
15	0·125	,,	[23]	27·1	,,	,,
25	0·5	,,	[100]	123·2	,,	,,
35	1·5	,,	[>4000]	944·0	,,	,,
3	0·075	0·248	[31]	32·2	0·00174	,,
13	0·125	,,	[39]	38·0	,,	,,
23	0·5	,,	[129]	173·0	,,	,,
33	1·5	,,	[>5000]	1323·0	,,	,,
1	0·075	0·148	[44]	54·0	0·00103	,,
11	0·125	,,	[50]	63·6	,,	,,
21	0·5	,,	[300]	289·5	,,	,,
31	1·5	,,	[>6500]	2218·0	,,	,,

0·325 cc. of fibrinogen solution was employed and 0·442 cc. of the former for 0·160 cc. of the latter). With the aid of these values of y and of the values of the product xy already selected the divergent values of x under consideration were recalculated; these are given within brackets in the last column of the tables and are seen to approach more nearly to the observed coagulation times than do the lower values obtained when the full amounts of thrombokinase present are taken.

[1] In this way the effective values of y in Table VIII were obtained.

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In order to study the effect of $CaCl_2$ upon the time of coagulation the experiments given in Tables IX and X were rearranged in Tables XI and XII respectively according to the effective amounts of thrombokinase present. This course was adopted because it was found, when searching for a relationship between the amounts of $CaCl_2$ (w) present and the calculated coagulation times (x), in presence of a fixed amount of thrombokinase (y), that the coagulation times, when plotted as abscissae with the corresponding amounts

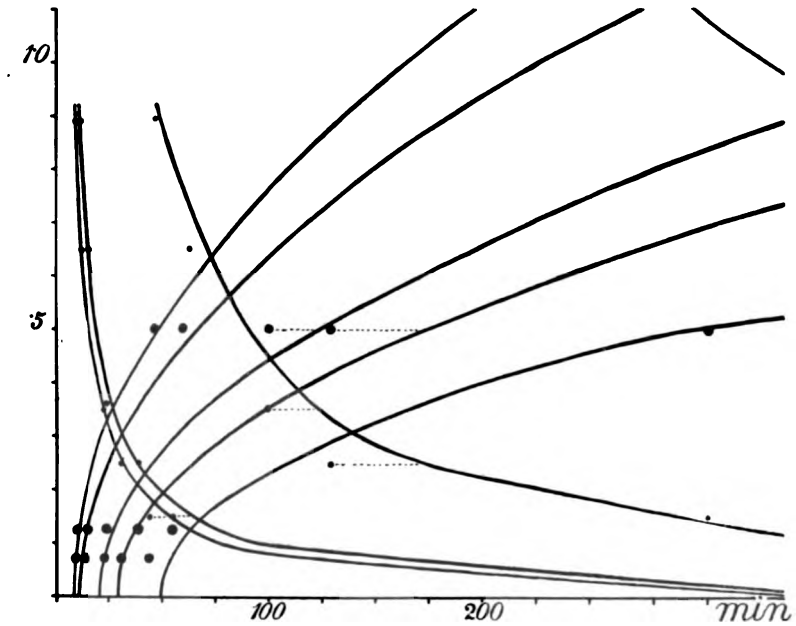

Fig. 4. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables IX and XI. Abscissae (x), time in minutes; ordinates, cc. of solution of thrombokinase (y) for hyperbolic curves (·) or of N/10 calcium chloride (w) for parabolic curves (•). The amount of fibrinogen solution employed in each experiment is 0·325 cc. The hyperbolic curve nearest to the axes corresponds to those experiments in which 0·075 cc. of decinormal calcium chloride solution was employed; the remaining hyperbolic curves to 0·125 cc., 0·5 cc. and 1·5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 0·897 cc. of thrombokinase solution was employed; the remaining parabolic curves to 0·648 cc., 0·348 cc., 0·248 cc. and 0·148 cc. respectively.

of decinormal $CaCl_2$ solution as ordinates, lay on a curve which appeared to be parabolic (Fig. 4 for Tables IX and XI; Fig. 5 for Tables X and XII). If parabolic the values of x must satisfy the equation

$$w^2 = ax - b \quad \text{.....(6)},$$

where a and b are constants. It will be observed that in each table five curves are investigated, corresponding to five different concentrations of

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thrombokinase and that four points on each curve are determined. If only a single curve had been studied a larger number of points could have been determined, but the advantage of investigating simultaneously a series of values of w would have been lost.

The manner in which a and b were determined is sufficiently indicated in the 5th and 6th columns of Tables XI and XII. Guidance in selecting

TABLE XII.

The experiments recorded in Table X, rearranged in a form suitable for calculating the values of a and b in the equation $w^2 = ax - b$. The observed coagulation times are given within brackets in the fourth column.

No. of experiment	w N/10 $CaCl_2$ solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	$x_{calculated}$ Time of coagulation (minutes)	$a=\frac{w_1^2-w^2}{x_1-x}$	$b=ax-w^2$
10	0·075	0·442	[10] 8·2	0·0362	0·290
20	0·125	,,	[7] 8·5		,,
30	0·5	,,	[12] 14·9	,,	0·291
40	1·5	,,	[780] 70·2	,,	0·290
8	0·075	,,	[10] 8·2	0·0362	,,
18	0·125	,,	[7] 8·5		,,
28	0·5	,,	[15] 14·9	,,	,,
38	1·5	,,	[780] 70·3	,,	,,
6	0·075	0·324	[13] 11·1	0·0265	,,
16	0·125	,,	[11] 11·5		,,
26	0·5	,,	[20] 20·3	,,	,,
36	1·5	,,	[780] 95·7	,,	,,
4	0·075	0·224	[20] 16·1	0·01835	,,
14	0·125	,,	[17] 16·7		,,
24	0·5	,,	[26] 29·4	,,	,,
34	1·5	,,	[1440] 138·7	,,	,,
2	0·075	0·124	[30] 29·1	0·01015	,,
12	0·125	,,	[39] 30·1		,,
22	0·5	,,	[56] 53·2	,,	,,
32	1·5	,,	[>6500] 250·0	,,	,,

final values for these constants is afforded by studying the effect, upon the recalculated values of x, produced by slightly varying the values first selected. When preliminary determinations of a and b had been made it was found that the values of a for each curve were roughly proportional to the amounts of thrombokinase employed, while the values of b were not far different for all the curves. It was therefore concluded that these indications were valid and values of a and b were accordingly selected which appeared best to fulfil the two conditions thus foreshadowed. It will be noted that, with the

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values selected, the calculated coagulation times agree with those actually found, within the limits of experimental error for the first three experiments of each group, but that a divergence between the two occurs when the equivalent of 1·5 cc. of decinormal $CaCl_2$ solution is employed (Exps. 39, 37, 35, 33 and 31, Table XI; Exps. 40, 38, 36, 34 and 32, Table XII) and that the divergence is more marked when 0·160 cc. of fibrinogen solution is employed (Table XII) than when 0·325 cc. is used (Table XI). In other words equation (6) fails when the concentration of $CaCl_2$ is increased relatively to the fibrinogen solution employed to such an extent that the ratio of the

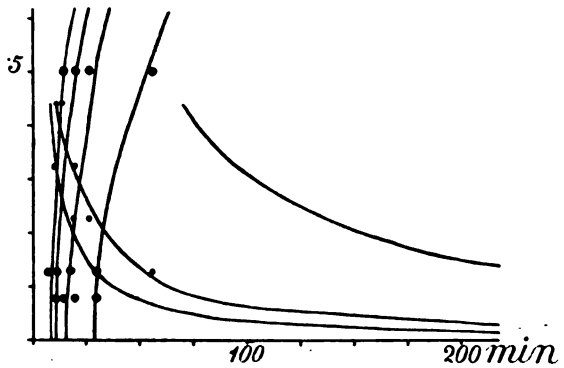

Fig. 5. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables X and XII. Abscissae (x), time in minutes; ordinates, cc. of solution of thrombokinase (y) for hyperbolic curves (·) and of N/10 calcium chloride (w) for parabolic curves (•). The amount of fibrinogen solution employed in each experiment is 0·160 cc. The hyperbolic curve nearest to the axes represents two curves, corresponding to those experiments in which 0·075 cc. and 0·125 cc. respectively of N/10 $CaCl_2$ were employed, the remaining hyperbolic curves to 0·5 cc. and 1·5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 0·442 cc. of thrombokinase solution was employed; the remaining parabolic curves to 0·324 cc., 0·224 cc. and 0·124 cc. respectively.

two reaches 4·6 (Table XI). With this ratio the observed values of x are two to four times as great as the calculated; when the ratio becomes 9·4 (Table XII) the former are roughly ten times as great as the latter.

Since a has been found to be proportional to y, in other words since

$$a = ky,$$

where k is a constant, equation (6) may be written

$$w^2 = kxy - b \quad \text{.....(7).}$$

It was further observed that the values of xy when the amount of $CaCl_2$ (w) was small were approximately proportional to the amount of fibrinogen

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solution (z) employed. It was therefore determined to introduce this relationship into the above equation by putting $k = p$, where p is a constant, and at the same time to express the constant b in the form of a multiple of p, namely pn, n of course being itself a constant. The equation thus becomes

$$w^2 = \frac{p}{z} xy - pn,$$

or

$$w^2 = p\left(\frac{xy}{z} - n\right) \quad \text{.....(8).}$$

If in the forty experiments at present under consideration approximate values of xy are first determined, in the manner indicated in Tables IX and X, and if from these the values of the constants p and n are next determined, it will be found that the estimated values of n lie close together for all the groups in both series of experiments, so that it seems legitimate to infer that n is independent of variations of z. This inference was adopted in calculating p and n in the experiments recorded in Tables IX to XII, the values selected being

$$n = 22{\cdot}12,$$
$$p_{0\cdot325} = 0{\cdot}00228,$$
$$p_{0\cdot160} = 0{\cdot}0131.$$

The mode of determining the two constants is illustrated in the series of experiments recorded in Tables XIII to XVI.

Having determined the values of the constants p and n from the approximate values of xy first reached according to the method indicated in Tables IX and X, the values of the product xy for varying concentrations of $CaCl_2$ may now be recalculated by means of equation (8). It thus becomes possible to determine the slight variations in the value of xy corresponding to the lower values of w, variations which may be too small to be determined satisfactorily by direct observation of coagulation times. It was in this way that the final values of xy, given in Tables IX and X were chosen, the effect of slight variations of p and n upon the calculated values of xy having been carefully studied before the values of these constants ultimately selected were adopted.

It may here be pointed out as a matter of practical convenience that the determination of p and n [equation (8)] is much simpler than the estimation of a and b [equation (6)] or of k and b [equation (7)]. As already mentioned n is independent of w, xy and z; p remains a function of z, but is independent of xy and w. If $w = 0$ in equation (8), a condition of experiment which

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cannot be realised experimentally however, xy becomes proportional to z and equation (2) (p. 516) is obtained, thus

$$\frac{xy}{z} - n = 0,$$

or

$$xy = nz.$$

It is thus seen that equation (2) is theoretically admissible only if w is relatively very small. If w is not sufficiently small to be negligible, then the complete formula

$$xy = \left(\frac{w^2}{p} + n\right) z$$

must be used.

Equation (6) is investigated in Tables XI and XII with a minimal addition of 0·075 cc. of decinormal calcium chloride solution (w) in each 0·5 cc. of fluid. The equation is valid until w falls to 0·03 cc.; below this amount a gradual lengthening of the coagulation time occurs (the amount of thrombokinase added remaining, as before, constant), the observed values of x being greater than the calculated values, so that equations (6) to (8) are no longer applicable. The complete w, x curve which is of the type shown in 2, Fig. 8 (p. 539) appears to be made up of two curves: (1) the parabolic curve already described; and (2) a flat curve, lying below 0·03 on the axis of ordinates, which as it proceeds to the right approaches the axis of abscissae. The latter curve, the accurate investigation of which is difficult, will not be further considered here; it appears to represent the extent to which thrombin has been produced from prothrombin in presence of relatively low percentages of calcium chloride. The form of the complete curve shows that it is not a hyperbola, such as is obtained when the inhibitory action of amboceptor upon complement is represented [Barratt 1913, 2 and 3]; the application of the equation for a hyperbola to the complete w, x curve is impossible.

The enquiry now presents itself, whether the influence of calcium chloride upon coagulation is concerned with the activity of thrombokinase or with that of thrombin. To this end the series of experiments recorded in Tables IX to XII were repeated, with thrombin, in the form of snake venom, in place of thrombokinase. In these experiments (Tables XIII and XIV) the values of xy were first determined as in the preceding series and it is seen that equation (1)

$$xy = \text{const.}$$

is applicable just as when thrombokinase is employed, allowance being made as before for the amount of thrombokinase already present in the fibrinogen

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solution employed, this being determined in terms of the thrombin solution employed. It will be observed, however, that in addition to the four groups given in each of the preceding Tables (IX and X) an additional group has been added in which no calcium chloride has been used, venom thrombin (and the same is true of thrombin produced from prothrombin by the action of thrombokinase) being capable of causing coagulation of fibrinogen when no calcium chloride has been added or when, by adding potassium oxalate,

TABLE XIII.

Determination of the coagulation time of a series of mixtures of venom and fibrinogen (0·325 *cc.*) *solutions in presence of* $CaCl_2$, *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment		N/10 $CaCl_2$ solution (cc.)	y Venom (*Echis carinatus*) solution, 1 in 900,000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	Value of xy selected	$x_{calculated}$ (minutes)
I	1	—	4·5	6	4·650	24·30	5·2
	11	—	0·6	35	0·750	,,	32·4
	21	—	0·115	102	0·265	,,	91·5
	31	—	0·045	120	0·195	,,	124·0
	41	—	0·015	152	0·165	,,	147·0
	51	—	—	510	0·150	,,	162·0
II	3	0·075	4·5	4	2·94 [4·604]	11·12	3·8
	13	,,	0·6	12	0·704	,,	15·8
	23	,,	0·035	50	0·209	,,	53·2
	33	,,	0·015	76	0·149	,,	74·7
	43	,,	0·005	122	0·119	,,	93·5
	53	,,	—	160	0·104	,,	107·0
III	5	0·125	4·5	4	2·94 [4·604]	11·25	3·8
	15	,,	0·6	12	0·704	,,	16·6
	25	,,	0·115	42	0·209	,,	53·9
	35	,,	0·045	76	0·149	,,	75·5
	45	,,	0·015	102	0·119	,,	94·5
	55	,,	—	160	0·104	,,	108·0
IV	7	0·5	4·5	5	2·94 [4·604]	14·19	4·8
	17	,,	0·6	18	0·704	,,	20·1
	27	,,	0·115	62	0·209	,,	67·9
	37	,,	0·045	100	0·149	,,	95·1
	47	,,	0·015	137	0·119	,,	119·2
	57	,,	—	182	0·104	,,	136·2
V	9	1·5	4·5	44	2·94 [4·604]	39·10	13·3
	19	,,	0·6	94	0·704	,,	55·5
	29	,,	0·115	330	0·209	,,	187·3
	39	,,	0·045	413	0·149	,,	262·3
	49	,,	0·015	640	0·119	,,	319·5
	59	,,	—	640	0·104	,,	376·0

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the calcium salt present in solution has been reduced to a point determined by the extremely low solubility of calcium oxalate. It is further to be noted that when no calcium chloride has been added the time of coagu'ation, after the addition of varying amounts of venom, is longer than is the case in presence of a small quantity of calcium chloride. For the present, however, attention will be given only to the IInd to Vth groups of Tables XIII and XIV, which alone can be compared with the preceding experiments with thrombokinase in Tables IX and X.

TABLE XIV.

Determination of the coagulation time of a series of mixtures of venom and fibrinogen (0·050 *cc.*) *solutions in presence of* $CaCl_2$, *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment		N/10 $CaCl_2$ solution (cc.)	y Venom (*Echis carinatus*) solution, 1 in 900,000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	Value of xy selected	$x_{calculated}$ (minutes)
I	2	—	4·5	4	4·560	7·30	1·6
	12	—	0·6	11	0·660	,,	11·0
	22	—	0·115	35	0·175	,,	41·7
	32	—	0·045	76	0·105	,,	69·6
	42	—	0·015	102	0·075	,,	97·2
	52	—	—	640	0·060	,,	122·0
II	4	0·075	4·5	4	0·453 [4·516]	1·73	3·8
	14	,,	0·6	5	0·453 [0·616]	,,	3·8
	24	,,	0·115	16	0·121	,,	14·3
	34	,,	0·045	25	0·061	,,	28·4
	44	,,	0·015	49	0·031	,,	35·8
	54	,,	—	103	0·016	,,	108·1
III	6	0·125	4·5	4	0·453 [4·516]	1·80	4·0
	16	,,	0·6	5	0·453 [0·616]	,,	4·0
	26	,,	0·115	16	0·121	,,	14·9
	36	,,	0·045	25	0·061	,,	29·5
	46	,,	0·015	55	0·031	,,	58·0
	56	,,	—	102	0·016	,,	112·4
IV	8	0·5	4·5	6	0·453 [4·516]	3·26	7·2
	18	,,	0·6	8	0·453 [0·616]	,,	7·2
	28	,,	0·115	27	0·121	,,	27·0
	38	,,	0·045	48	0·061	,,	53·5
	48	,,	0·015	93	0·031	,,	105·0
	58	,,	—	180	0·016	,,	204·0
V	10	1·5	4·5	31	0·453 [4·516]	15·75	34·8
	20	,,	0·6	40	0·453 [0·616]	,,	34·8
	30	,,	0·115	159	0·121	,,	130·1
	40	,,	0·045	248	0·061	,,	258·0
	50	,,	0·015	510	0·031	,,	507·4
	60	,,	—	>2500	0·016	,,	985·0

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When calcified venom was employed the lowest values of xy first selected in the two series (11·12 and 1·73 were ultimately chosen) were found to be very nearly in the ratio of the amounts of fibrinogen solution added (0·325 cc. and 0·050 cc. respectively), indicating that these groups conformed approximately to equation (2)

$$xy = nz.$$

It was also found that the thrombin of venom was effective until an amount equivalent to 2·94 cc. of the venom solution for the first series (Table XIII) and 0·453 cc. for the second series (Table XIV) was used; increase beyond this amount did not cause a corresponding diminution of the coagulation

TABLE XV.

The experiments recorded in Table XIII rearranged in a form suitable for calculating the values of p and n in the equation $w^2 = p\left(\frac{xy}{z} - n\right)$. *The observed coagulation times are given within brackets in the fourth column.*

No. of experiment	w N/10 $CaCl_2$ solution (cc.)	y Venom solution (*Echis carinatus*), 1 in 300,000 (cc.)	$x_{calculated}$ Time of coagulation (minutes)	xy	$p = \frac{w_1^2 - w^2}{\frac{x_1y_1}{z} - \frac{xy}{z}}$	$n = \frac{xy}{z} - \frac{w^2}{p}$
3	0·075	2·94	[4] 3·8	11·12	0·026	34·01
5	0·125	,,	[4] 3·8	11·25		34·04
7	0·5	,,	[5] 4·8	14·19	,,	34·07
9	1·5	,,	[44] 13·3	39·10	,,	33·90
13	0·075	0·704	[12] 15·8	11·12		34
15	0·125	,,	[12] 16·6	11·25	,,	,,
17	0·5	,,	[18] 20·1	14·19	,,	,,
19	1·5	,,	[94] 55·5	39·10	,,	,,
23	0·075	0·209	[50] 53·2	11·12		,,
25	0·125	,,	[42] 53·9	11·25	,,	,,
27	0·5	,,	[62] 67·9	14·19	,,	,,
29	1·5	,,	[330] 187·3	39·10	,,	,,
33	0·075	0·149	[76] 74·7	11·12		,,
35	0·125	,,	[76] 75·5	11·25	,,	,,
37	0·5	,,	[100] 95·1	14·19	,,	,,
39	1·5	,,	[413] 262·3	39·10	,,	,,
43	0·075	0·119	[122] 93·5	11·12		,,
45	0·125	,,	[102] 103·2	11·25	,,	,,
47	0·5	,,	[137] 119·2	14·19	,,	,,
49	1·5	,,	[640] 319·5	39·10	,,	,,
53	0·075	0·104	[160] 107·0	11·12		,,
55	0·125	,,	[160] 108·0	11·25	,,	,,
57	0·5	,,	[182] 136·2	14·19	,,	,,
59	1·5	,,	[640] 376·0	39·10	,,	,,

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time. In these respects the experiments with venom resemble those already made with thrombokinase and are in agreement with the series shown in Table VIII.

The experiments with calcified venom were now, like the series given in Tables IX and X, rearranged (Tables XV and XVI) for convenience of ascertaining whether or no equation (8)

$$w^2 = p\left(\frac{xy}{z} - n\right)$$

was applicable. It was found that (except in the case of the last group of experiments shown in Tables XIII and XIV) with very slight changes in the values of xy first chosen, values of p and n (given in Tables XV and XVI) could be selected such that the coagulation times recalculated therefrom, given in the tables, were in agreement with the observed periods, within the limits of experimental error. The curves for Tables XIII and XV are given in Fig. 6; those for Tables XIV and XVI in Fig. 7.

It thus appears that in presence of calcium chloride both series (Tables IX to XII and XIII to XVI respectively) behave alike, the same equation being applicable equally to the experiments made with thrombokinase and thrombin. The inhibitory effect of this salt upon fibrin production is therefore manifested in respect of the interaction of thrombin and fibrinogen, the action of thrombokinase upon prothrombin being unaffected. Calcium chloride moreover in the concentrations employed produces from the first retardation of the process of coagulation of fibrinogen by means of thrombin.

If the constants p and n for the experiments with thrombokinase and snake venom are compared as shown below, it will be observed that a striking

	n	Fibrinogen solution employed in each experiment	p 0·325 cc.	p 0·160 cc.	p 0·050 cc.
Thrombokinase (testis of rabbit)	22·12		0·00228	0·0131	—
Thrombin (venom of *Echis carinatus*) ...	34·00		0·026	—	0·0080

difference occurs between the two in respect of the constant p. In the former experiments p increases with diminution of the amount of fibrinogen solution employed; in the latter the reverse occurs. This is readily explicable on the assumption that different kinds of thrombin are present in the two series of experiments. In reality the conditions of experiment are unavoidably complex, for the thrombin present comes from four sources: (1) that produced

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by the action of thrombokinase, contained in relatively small amount in the blood plasma of the fowl, upon fowl's prothrombin in presence of calcium chloride; (2) that produced by the action of thrombokinase from the testis of the rabbit upon fowl's prothrombin; (3) that produced by the action of thrombokinase, contained in relatively small amount in snake venom (cf. p. 540), upon fowl's prothrombin; and (4) that contained in snake venom.

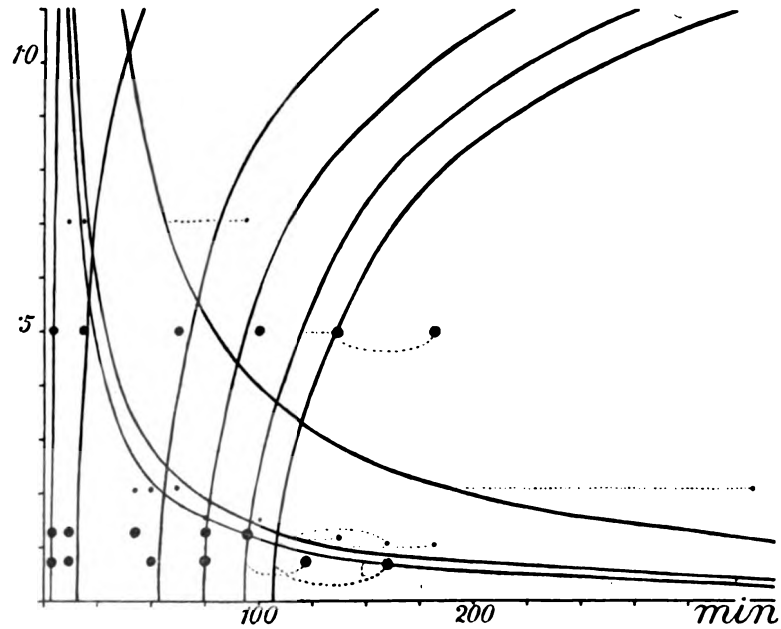

Fig. 6. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables XIII and XV. Abscissae (x), time in minutes; ordinates, cc. of 1 in 300,000 solution of venom (y) for hyperbolic curves (·) or of N/10 $CaCl_2$ (w) for parabolic curves (•). The amount of fibrinogen solution employed in each experiment is 0·325 cc. The hyperbolic curve nearest to the axes represents two curves, corresponding to those experiments in which 0·075 cc. and 0·125 cc. respectively of N/10 $CaCl_2$ solution were employed, the remaining hyperbolic curves to 0·5 cc. and 1·5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 2·94 cc. of venom solution was employed; the remaining parabolic curves to 0·704 cc., 0·209 cc., 0·149 cc., 0·119 cc. and 0·104 cc. respectively.

The chief sources of thrombin are (2) and (4). The enquiry naturally arises, Is the thrombin produced from fowl's prothrombin independent of the source of the thrombokinase employed? The difference in the values of p in the two series of experiments appears to indicate that the thrombin of snake venom is different from that produced by the action of the thrombokinase of the rabbit's testis upon fowl's prothrombin; thrombin produced by the action of the thrombokinase of snake venom upon fowl's prothrombin is

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present in too small an amount to enable a safe comparison with the other thrombins under consideration to be made. That the thrombin derived from fowl's prothrombin by the action of fowl's thrombokinase (both contained in the fibrinogen solution employed)—the only auto-thrombin present in these experiments—is not similar to snake venom thrombin is suggested by the circumstance that the more marked divergences between observed and calculated values of x are seen in those experiments in Tables XIII and XIV in which the amount of thrombokinase present is low and the amount of the former variety of thrombin is relatively high, e.g. Exps. 53 and 43 of the second group in Table XIII and similar experiments in adjoining groups.

TABLE XVI.

The experiments recorded in Table XIV rearranged in a form suitable for calculating the values of p and n in the equation $w^2 = p\left(\frac{xy}{z} - n\right)$. The observed coagulation times are given within brackets in the fourth column.

No. of experiment	w N/10 $CaCl_2$ solution (cc.)	y Venom solution (*Echis carinatus*), 1 in 300,000 (cc.)	$x_{calculated}$ Time of coagulation (minutes)	xy	$p = \frac{w_1^2 - w^2}{\frac{x_1 y_1}{z} - \frac{xy}{z}}$	$n = \frac{xy}{z} - \frac{w^2}{p}$
2	—	0·453	[4] 3·7	1·70	0·0080	34·00
4	0·075	,,	[4] 3·8	1·73		34·00
6	0·125	,,	[4] 4·0	1·80	,,	33·99
8	0·5	,,	[6] 7·2	3·26	,,	33·95
10	1·5	,,	[31] 34·8	15·75	,,	33·85
14	0·075	,,	[5] 3·8	1·73		34
16	0·125	,,	[5] 4·0	1·80	,,	,,
18	0·5	,,	[8] 7·2	3·26	,,	,,
20	1·5	,,	[40] 34·8	15·75	,,	,,
24	0·075	0·121	[16] 14·3	1·73		,,
26	0·125	,,	[16] 14·9	1·80	,,	,,
28	0·5	,,	[27] 27·0	3·26	,,	,,
30	1·5	,,	[159] 157·4	15·75	,,	,,
34	0·075	0·061	[25] 28·4	1·73		,,
36	0·125	,,	[25] 29·5	1·80	,,	,,
38	0·5	,,	[48] 53·5	3·26	,,	,,
40	1·5	,,	[248] 258·0	15·75	,,	,,
44	0·075	0·031	[49] 35·8	1·73		,,
46	0·125	,,	[55] 58·0	1·80	,,	,,
48	0·5	,,	[93] 105·0	3·26	,,	,,
50	1·5	,,	[510] 507·4	15·75	,,	,,
54	0·075	0·016	[103] 108·1	1·73		,,
56	0·125	,,	[102] 112·4	1·80	,,	.
58	0·5	,,	[180] 204·0	3·26	,,	.
60	1·5	,,	[>2500] 985·0	15·75	,,	,

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The same process of reasoning applies when, for the latter variety of thrombin, that produced from fowl's prothrombin by the action of rabbit's thrombokinase (testis) is substituted (Tables IX and X). It is interesting to observe that in the former case the observed coagulation times for low amounts of thrombokinase are longer than the calculated, while in the latter case the reverse occurs.

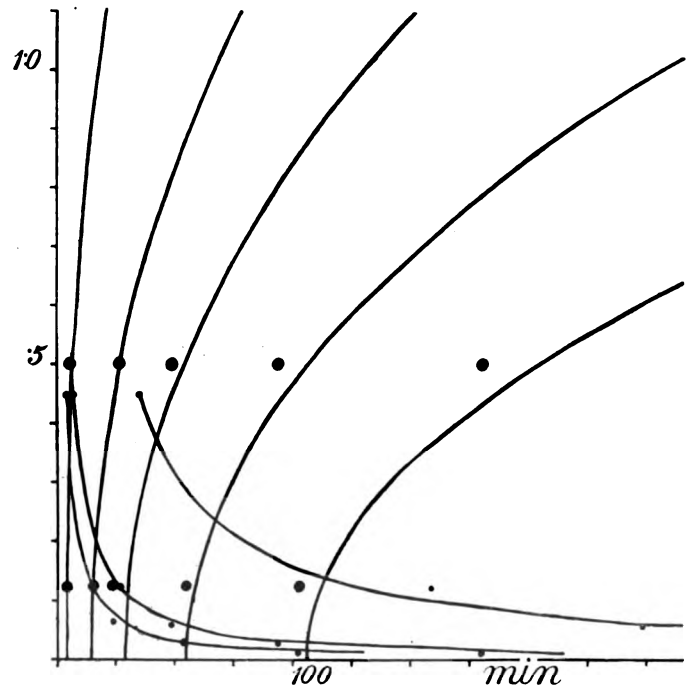

Fig. 7. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables XIV and XVI. Abscissae (x), time in minutes; ordinates, cc. of 1 in 300,000 solution of venom (y) for hyperbolic curves (•) or of N/10 $CaCl_2$ (w) for parabolic curves (●). The amount of fibrinogen solution employed in each experiment is 0·050 cc. The hyperbolic curve nearest to the axes represents two curves, corresponding to those experiments in which 0·075 cc. and 0·125 cc. respectively of N/10 $CaCl_2$ were employed, the remaining hyperbolic curves to 0·5 cc. and 1·5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 0·453 cc. of venom solution was employed; the remaining parabolic curves to 0·121 cc., 0·061 cc., 0·031 cc. and 0·016 cc. respectively.

It has already been pointed out that equation (8)

$$w^2 = p\left(\frac{xy}{z} - n\right)$$

only applies so long as the concentration of calcium chloride does not exceed a limit, which is dependent upon the concentration of fibrinogen. When this limit has been passed, the retardation of coagulation becomes greater than

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that calculated from equation (8): in other words, the calculated values of xy are lower than the observed values (cp. the last group of experiments in Tables IX, X, XIII and XIV). In the experiments made with thrombokinase (testis of rabbit) the observed retardation was greater when the concentration of fibrinogen was diminished (0·325 cc. was used in each experiment in Table IX; 0·160 cc. in Table X); with thrombin obtained from venom the observed retardation was more marked when the concentration of fibrinogen was increased (0·325 cc. in Table XIII; 0·050 cc. in Table XIV). This circumstance, which is indicated by the increase of p with diminution of z (fibrinogen) in the former experiments and its decrease with increase of z in the latter, is in its ultimate aspect an index of the physical conditions of experiment. Presumably with an auto-thrombin derived from the same source as the fibrinogen employed, p would increase with decrease of z, while with a hetero-thrombin a smaller increase or an actual decrease would occur.

So far we have only considered those experiments in which calcified venom has been used. If no calcium chloride is employed a retardation of the time of coagulation occurs as is exhibited in the first group of experiments in Tables XIII and XIV; with amounts of calcium chloride considerably below 0·075 cc. in each experiment a less marked retardation occurs. The complete w, x curve for snake venom thrombin is therefore of the type shown in 1, Fig. 8. Any attempt to apply the equation for a hyperbola to this curve is unsuccessful, as is obvious from the aspect of the curve. It appears to consist of two parts: (1) the parabolic curve just described; and (2) a low descending curve. The complete curve thus presents a general resemblance to the former curve furnished by thrombokinase (2, Fig. 8). In both an upper parabolic curve is joined below by a lower flat curve. But here the resemblance ceases, the lower curve obtained with thrombokinase approaching but not touching the axis of abscissae, while that obtained with venom thrombin cuts this axis; the former as already mentioned is due to the action of thrombokinase upon prothrombin in presence of minimal amounts of calcium chloride, the latter is due to an accelerator action of calcium chloride upon venom thrombin, an effect which is produced with very low concentrations of the calcium salt and is speedily followed by the inhibitory

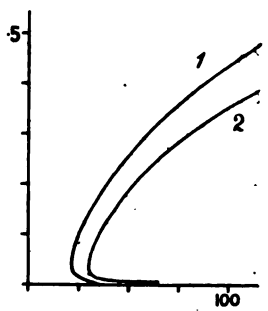

Fig. 8. Type curves. Abscissae, time in minutes; ordinates, calcium chloride. (cc.)

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action investigated above. It is important to distinguish this action of calcium chloride in low concentration upon venom thrombin from the action exerted upon thrombokinase in presence of prothrombin contained in the fibrinogen solution employed. In point of fact the venom solution contained thrombokinase, for upon heating to 100° for five minutes a solution was obtained free from thrombin, but containing enough thrombokinase to yield in presence of prothrombin and calcium chloride an amount of thrombin equal to about one-twentieth of that originally present, a quantity which if applied as a correction in Tables XIII and XIV would cause a very slight decrease of some of the calculated coagulation periods. Evidence is thus afforded of the increased coagulative activity of venom thrombin in presence of minimal amounts of calcium chloride. Presumably the same effect under similar conditions of experiment is also produced with thrombin derived from the action of thrombokinase (testis of rabbit) upon fowl's prothrombin.

Before concluding this article further reference may be made to the differences between observed and calculated times of experiment exhibited in the tables. Two sources of these differences, namely (1) that due to the circumstance that thrombin has a maximum effective value and (2) that caused by the concentration of calcium chloride exceeding a limiting value, have already been considered. In addition a certain amount of irregularity is observable, which appears to be at present unavoidable in experiments upon coagulation. Although it is not possible to deal with all the sources of experimental error, some of which appear still to elude detection, while others, e.g. decomposition and evaporation, are variable and especially likely to become effective when the coagulation period is long, nevertheless two sources of irregularity of result to which special attention has been given may be referred to here, namely that due to error of observation of the completion of coagulation and that caused by imperfect measurement of the amounts of the various solutions employed.

The former is a serious difficulty when quantitative work upon coagulation is first undertaken. The problem is to judge when coagulation is complete, that is to say when the whole of the fibrin has separated out. The indications that this has occurred are that the clot has reached (1) its maximum consistence and (2) its maximum opacity; the latter, however, does not appear to be so valuable an indication as the former. Since there is no sharp end point, precise measurement of coagulation time is not possible. Even with the training of observation resulting from the repeated performance of serial experiments, in which the degrees of coagulation in mixtures of slightly

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different composition are constantly being compared, the measurements made must be regarded as only approximate. Difficulty is sometimes caused by coagulation being irregular, fibrin separating out to form a framework of strands of varying length and thickness, the fibrils being no longer distributed uniformly throughout the clot; this condition which is occasionally troublesome has been met with chiefly when the amount of fibrinogen has been small, but it sometimes appears when relatively large amounts of fibrinogen have been employed. To judge of the consistence of a clot it is necessary to agitate or shake the tube in which the clot is contained. If this is not done gently a soft clot may be detached before complete separation of fibrin has occurred; contraction of the clot may then take place followed by the appearance of a second coagulum, usually of soft consistence, in which case the determination of the coagulation time cannot be made with much approach to precision.

A second source of error arises from the difficulty of delivering accurately the small amounts of fluid which must necessarily be dealt with when large numbers of experiments have to be made with limited quantities of fibrinogen solution. It must be borne in mind that it is not the amount of fluid which is *contained* in the measuring pipette, but the quantity which is actually *delivered*, which requires accurate measurement. Consequently the amounts actually delivered must be determined and also the extent of the variations occurring between successive deliveries from the same scale mark. When this has been done the degree in which the time of coagulation is likely to be affected by unavoidable error of delivery can be estimated with the aid of the data given in the tables. The tendency of fluid to adhere to glass in the neighbourhood of the mouth of a pipette may be overcome by coating the outside of the end of the pipette with hard paraffin. Any greasy material present on the inner surface of the pipette interfering with filling and delivery is best removed by heating to a temperature just sufficient to cause any organic matter present to become charred; the inner surface of the pipette is then readily wetted by water. In performing a series of experiments each pipette should be used exclusively for one constituent of the mixture of liquids employed; if used for several constituents in succession, very careful cleaning may not suffice to avoid contamination. In practice, when performing serial experiments similar to those recorded in the tables, it will be found convenient to prepare a series of dilutions of calcium chloride such that an equal amount of fluid is delivered in each experiment, the same pipette, filled to the same scale mark, being used for each tube, proceeding from lower

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to higher concentrations, and being washed out several times with each liquid of higher concentration before commencing to deliver. The solutions of thrombokinase were similarly prepared and dealt with. In order to destroy traces of thrombokinase remaining adherent to the inner surface of the tubes after coagulation has been completed, the latter were, after being previously cleaned, heated just below redness before being again employed.

In conclusion I desire to express my indebtedness to Dr C. J. Martin for the valuable assistance he has given me in connection with this research.

Summary.

(1) A given amount of thrombokinase, in presence of calcium chloride and of a sufficient amount of prothrombin, will produce a definite quantity of thrombin, which is independent of the actual concentration of prothrombin.

(2) In experiments with thrombokinase the coagulation time is concerned with the action of thrombin upon fibrinogen.

(3) The action of thrombin (y) upon fibrinogen (z) when a decinormal calcium chloride solution is present to the extent of 0·05 cc. in a total volume of 0·5 cc. proceeds approximately according to the equation

$$xy = nz,$$

where x = coagulation time and n is a constant.

(4) The effect of adding decinormal calcium chloride solution, in amounts exceeding 0·05 cc. per 0·5 cc. of fluid, to (1) snake venom thrombin or (2) a mixture of thrombokinase and prothrombin is to cause retardation of coagulation of fibrinogen. If the coagulation times (x) are plotted against the amounts of calcium chloride employed, the points so obtained are found to lie on a parabola given by the equation

$$w^2 = ax - b,$$

so long as the amount of calcium chloride lies below a critical limiting value, beyond which this equation is no longer valid. Of the two constants in the equation a is proportional to the amount of thrombokinase present; b is a function of z, whose form has not been determined.

(5) The above action of thrombin upon fibrinogen takes place according to the general equation

$$w^2 = p\left(\frac{xy}{z} - n\right),$$

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where n is a constant, independent of w, xy or z, and p is a function of z, not yet determined.

(6) Minimal quantities of calcium chloride (i.e. amounts not exceeding 0·05 cc. of a decinormal solution, contained in a total volume of fluid amounting to 0·5 cc.) accelerate the coagulant activity of thrombin furnished by the venom of *Echis carinatus*.

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L. ON THE EFFECTS OF VARIOUS SUBSTANCES (ELECTROLYTES, NON-ELECTROLYTES, ALKALOIDS, ETC.) UPON THE UREASE OF SOY-BEAN.

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Musculus [1874] was the first to discover the ferment urease, which is capable of decomposing urea into NH_3 and CO_2, in urine. Shibata found urease in *Aspergillus niger* [1904], and Kikkoji in *Cortinellus edodes* [1907]. Takeuchi was the first to discover urease in soy-bean in considerable amount [1909]. Various workers in succession [Keisel, 1911; Zemplén, 1912; and Annet, 1914] succeeded in finding urease in varying amounts in the seeds of various higher plants (wheat, lupin, some Indian seeds, etc.). Subsequently Marshall [1913, 1 and 2] and Plimmer and Skelton [1914] used the urease of soy-bean to estimate urea in urine, blood, etc., with extreme accuracy. These investigations were followed by the study of the mode of action of urease and the conditions which facilitate or retard its action.

Takeuchi endeavoured to ascertain the extent of the influence of salts, acids and caustic soda on urease. But we find his data of the concentration of the substances used by him too high, owing to the lack of an accurate method of estimation.

Armstrong and Horton [1912] and Armstrong, Benjamin and Horton [1913] studied the influence of various substances upon urease. They found that urea at a concentration of more than M/5 depressed the development of NH_3, and all acids, even such relatively weak acids as carboxylic acids, acted on urease in an inhibitory manner, with the exception of amino acids and carbon dioxide which accelerated the action of urease. Boric acid was

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inhibitory at M/50 and phenol at M/5. These observers also found that alcohols, aldehyde and glucose act as inhibitors; as regards alcohols, the lower their solubility, the greater was their inhibitory effect.

Marshall [1914] found that the velocity of action of urease is dependent on the hydrogen or hydroxyl ion concentration within rather narrow limits. Hydrochloric acid and caustic soda in sufficient amounts inhibited or destroyed urease. Ethyl alcohol had a moderate inhibitory effect but had apparently a slight destructive action upon urease. It markedly retarded urease action in concentrations over 20 volumes per cent.

Armstrong and his collaborators, and Marshall, used the water extract of soy-bean powder as urease. This extract contains a certain amount of buffer substances, such as albumin, which disturb the accurate neutralisation of the extract by means of titration. This was recognised more or less by these authors, and when they desired to ascertain the effect of dilute acids upon urease, these acids were mixed with urea and urease in the digestion bottle. It was found that the acids added in small quantities may be neutralised after a short time by the development of NH_3.

To avoid the difficulty due to certain impurities, van Slyke and Cullen [1914, 2] tried successfully to obtain urease in a purer state by precipitating the water extract of soy-bean powder with acetone and drying the precipitate in vacuo over sulphuric acid. This dried precipitate, dissolved in distilled water before use, was used as urease. Van Slyke and his co-workers [van Slyke and Cullen, 1914, 1; van Slyke and Zacharias, 1914] also studied the effect of hydrogen ion concentration upon urease, altering the former as required by means of a phosphate mixture. They regarded the urease reaction as proceeding in two stages characterised by different velocities, the first being the combining velocity (c) of the urease and substrate, and the second the decomposing velocity (d) of the combined substance into NH_3 and CO_2. The more alkaline the reaction of the medium, the greater was c, but d was maximal at the neutral reaction and less when the reaction was more alkaline or more acid. The total action of urease was depressed at the slight acid reaction of $P_H = 6{\cdot}07$ and reached its maximum when P_H was close to 7. The authors ascribed the depressing effect of the product of the action to its hydrogen ion concentration, because the further addition of ammonia to the medium is no longer able to depress the urease action, since the hydrogen ion concentration reaches a certain limit and remains constant in spite of the increase of ammonia. Neutral salts, glucose and ethyl alcohol retarded c and at a high osmotic pressure retarded d as well.

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Armstrong and Horton [1912] found that the velocity of action of urease proceeds independently of the amount of the substrate remaining unchanged, provided that the product with retarding effects on urease is neutralised.

Van Slyke and his collaborators introduced a formula for general use in enzyme action, making use of c and d. This formula is as follows:

$$t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right).$$

They recommended this formula as the most accurate one because it corresponded exactly to the experimental data. They alleged that urease was not a contact catalyst but formed an intermediate labile compound with urea. They insisted that the action of urease was in accordance with the law of mass-action.

My experiments were directed towards ascertaining whether the hydrogen ion concentration alone is adequate to account for the effect of acids upon urease, as the custom is growing of stating results on enzyme action in terms of hydrogen ion concentration. This tendency has been increased by the work of Michaelis and his co-workers, and of Sörensen, etc. If enzymes are colloids and the reactions take place at the surface of colloids, it is reasonable to consider other physico-chemical properties at the same time as osmotic pressure and hydrogen ion concentration. Among the physico-chemical properties surface tension is the first to come into consideration. According to the Gibbs-Thomson theorem, substances which lower the surface tension of solutions accumulate at the surface of the second phase, e.g. at the boundary between air and solution or at the interface between suspended particles of solid and the solution, or between droplets of an immiscible fluid and the solution. Thus substances which actively affect surface tension may influence in one or other direction the actions which have some relation to surface. They may have an effect on enzyme particles in the sense of diminution or increase of active surface, i.e. aggregation or dispersion. They may also have effects on diffusion, absorption, adsorption, internal friction, etc. (see Traube's reports). To solve these questions various acids, alcohols, etc., which alter the surface tension of the solvent, were examined. I also endeavoured to ascertain whether the depressing effect of ammonium carbonate on urease should be ascribed to the hydroxyl ion concentration alone or in part to the reversible reaction of the hydrolysis of urea.

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Experimental Part.

Methods.

Urease solution was mixed with urea in digestion bottles. After some hours, acid of considerable concentration was added in order to stop the urease action instantaneously. The ammonia developed on subsequent addition of sodium carbonate was driven off by means of an air current free from ammonia and caught in decinormal sulphuric acid. The absorbed ammonia was estimated by the usual method of titration by N/10 caustic soda.

The experiments were always carried out at room temperature. The digestion bottles were placed in a large wooden water bath in a position in the laboratory where the change of air temperature was as small as possible. During the period of several months in which the experiments were carried out, the temperature of the bath varied between 19° and 21°. The change of temperature during each experiment was very slight.

As digestion bottles I used the high cylinders used by Plimmer in his work on urea estimation by means of urease. The inner diameter of these cylinders is about 4·2 cm., and their height about 40 cm. Cylinders were selected which had the same thickness of wall approximately.

The Allihn bottles, into which the developed ammonia was led to combine with decinormal sulphuric acid, are about 17 cm. high, with a content of 400 cc. The tubes which lead the ammonia to the sulphuric acid have six small holes at the closed end, each hole having a diameter of about 1·5 mm. Air with ammonia is bubbled through these holes into the sulphuric acid. The faster the air containing ammonia is drawn through these holes, the smaller are the bubbles and the more accurate is the ammonia estimation, as Folin, van Slyke and Plimmer ascertained.

In my experiments the urea was used in a concentration of 0·15 to 0·12 % of the whole mixture in the digestion bottle. This concentration of urea is the most favourable for urease action (van Slyke).

In order to check the urease action instantaneously sufficient N/5 sulphuric acid was added to the bottles to make the whole at least decinormal after neutralisation of the ammonia present. This checking action was complete in half an hour, but the bottles were always left for one or two hours. The mixture was then neutralised by the addition of 2N caustic soda and crystalline sodium carbonate added to make a 4 % solution. Folin's aeration was carried out in the water bath at a temperature of 40°.

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The N/10 sulphuric acid which was to catch the ammonia was made up with distilled water to 250 cc. and alizarin red was used as indicator. To prevent frothing a small quantity of lubricating oil was poured into the bottles. The escape of ammonia was prevented during the urease action by the use of rubber corks or ordinary corks and wax. Unless specially mentioned the total quantity of acting medium was always 50 cc. Each digestion bottle contained 5 cc. of 1·5 % solution of urea, with the exception of one series of experiments to be mentioned later in the experimental part. If the action of urease is complete with this amount of urea, then theoretically 25 cc. of N/10 ammonia should be evolved.

Two different preparations of urease were used, one being the 1–10 % water extract of soy-bean powder. This powder was digested carefully in distilled water, left half an hour, with occasional stirring, and filtered through linen. Only the fresh filtrate was used. The other solution was that of precipitated urease prepared by van Slyke's method. This solution was always made immediately before use and the undissolved flocculent substance was filtered off.

The reaction of the water extract of soy-bean was faintly acid compared with that of the standard neutral solution of Prideaux [1912], alizarin red being used as indicator. This extract did not turn phenolphthalein red, as Armstrong stated. Marshall, however, found the water extract of soy-bean alkaline to methyl orange.

0·1 cc. of N/10 ammonia was evolved from 10 cc. of 10 % water extract of soy-bean after standing for 20 hours at room temperature. This correction is made in the experimental data.

The reaction of the solution of the precipitated urease was found by van Slyke to be acid, but in my preparation it was quite neutral.

In order to make certain of my technique of ammonia estimation I carried out the following preparatory experiments.

I made up a N/5 NH_4Cl solution and by Kjeldahl's method I found 19·6 cc. N/10 ammonia in 10 cc. of this NH_4Cl solution. Folin's method was carried out in the water bath at a temperature of 15° for three hours, and the amount of N/10 NH_3 obtained was only 16·98 and 18·20 cc. instead of 19·6 cc.

In the next experiment Folin's distillation of ammonia was carried out in the water bath at a temperature of 40° for an hour, an hour and a half, and two hours respectively. The amounts of N/10 NH_3 obtained were 19·65, 19·69 and 19·66 cc. respectively. We know thus that all the ammonia was

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driven out under these conditions within an hour. In all the following experiments the distillation lasted for an hour and a half to two hours.

To check the urease action instantly, I always used sulphuric acid in my work, and avoided the use of heat. It is known that boiling of urea solution with acid or alkali develops ammonia to a small extent [see Armstrong and Horton, 1912].

To 5 cc. of 1 % bean extract N/5 H_2SO_4 was added to make N/10 and N/50 solution *in toto*. The acid was allowed to act on urease fifteen minutes, then the mixture was neutralised with N/5 NaOH. Urea was then added, the mixture was kept at room temperature the whole night, and NH_3 was determined in this mixture exactly as described previously. The control mixture without acid developed 23·83 cc. of N/10 NH_3. The urease inhibited by N/10 H_2SO_4 developed only 0, 0·03, 0·11, 0·05 cc., an average of 0·05 cc. of N/10 NH_3, and urease inhibited by N/50 H_2SO_4 developed only 0·06, – 0·02 cc., an average of 0·02 cc. of N/10 NH_3. Notwithstanding this fact, in order to check urease action, I always added to the digestion bottles N/5 H_2SO_4 so as to make the whole mixtures stronger than N/10 and left them for half an hour to one hour.

Urease mixed with 4 % crystalline sodium carbonate developed no ammonia at 40° in several hours.

(1) *Effects of acids on urease.*

Marshall found that the velocity of urease action is dependent upon the hydrogen or hydroxyl ion concentration within rather a narrow limit. Van Slyke found that slight acidity ($P_H = 6·07$) exercised a depressing effect on urease action and that the velocity reached its maximum at the neutral point ($P_H = 7$).

I made my first experiments with a water extract of soy-bean as did Armstrong and Marshall. Before use the bean extract had been neutralised with N/20 NaOH, being compared with the standard solution of Prideaux.

The acids employed were prepared by titration against N/10 NaOH, using phenolphthalein as an indicator. Only phosphoric acid was prepared by weighing from Merck's pure preparation, and this was estimated by weight after being converted into magnesium pyrophosphate.

The first experiment was commenced with sulphuric acid; the acid was mixed in digestion bottles with 5 cc. of 1·5 % urea solution and 10 cc. of a 2·5 % extract of the bean and left for nearly 20 hours at room temperature. Urease action was then checked and NH_3 estimated.

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We see from this experiment that sulphuric acid is capable at a concentration of N/100 and N/500 of totally inhibiting urease, while at a concentration of N/1000 and N/1500 it has practically no effect.

In order to ascertain to what extent the enzyme was inactivated by the prolonged action of acid, the urease and acid were mixed, left for some time and then neutralised with NaOH before the addition of urea.

TABLE I.

H_2O in cc.	H_2SO_4 in cc.	Conc. of acid	N/10 NH_3 developed in 17 hrs in cc.	
35	—	—	24·21	24·14
35	—	—	24·06	
30	N/10 5	N/100	0·02	0·12
30	N/10 5	N/100	0·11	
30	N/50 5	N/500	0·29	0·25
			0·20	
10	N/500 25	N/1000		24·08
10	N/750 25	N/1500		23·98

Sulphuric acid was allowed to act on urease for 16·5 hours, then neutralised, mixed with urea and left for 22·5 hours at room temperature. The urease action was then checked and the NH_3 estimated. The control urease developed 24·42 cc. of N/10 NH_3. N/500 sulphuric acid was found to have totally inactivated the urease; and N/1000 sulphuric acid had almost completely inactivated the urease, only 0·5 cc. of N/10 NH_3 being formed.

In order to find out the lower limit of the inactivating effect of sulphuric acid the experiment recorded in Table II was carried out. The columns of this table read from left to right show the order in which the various substances were added to the digestion bottles. In every experiment the procedure was carried out as Tables I and II.

TABLE II.

H_2O in cc.	1 % bean extract in cc.	N/200 H_2SO_4 in cc.	Conc. of acid	Added after 17 hours: H_2O in in cc.	Added after 17 hours: N/200 NaOH in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 20·5 hrs. in cc.	
40	10	—	—	10	—	5	24·05	24·16
40	,,	—	—	,,	—	,,	24·27	
30	,,	10	N/1000	—	10	,,		0·10
33	,,	7	N/1429	3	7	,,		0·62
35	,,	5	N/2000	5	5	,,		13·86
36	,,	4	N/2500	6	4	,,		23·98
37	,,	3	N/3300	7	3	,,	24·12	24·03
37	,,	,,	,,	,,	,,	,,	23·93	
38	,,	2	N/5000	8	2	,,	24·15	24·04
38	,,	,,	,,	,,	,,	,,	23·93	

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When the added sulphuric acid was not neutralised by the NH_3 developed, a markedly depressing effect of N/2000 H_2SO_4 in 17 hours was observed, N/2500 acid showed some depressing effect, whilst N/3330 had no effect.

It is to be noticed here that acids, whether organic or inorganic, caused in urease solution an opacity or fine flocculent precipitation. Even N/5000 H_2SO_4 caused a marked opacity, which disappeared on neutralisation. If this neutralised mixture of acid and urease still contains some effective urease, and consequently ammonia is developed, the mixture goes into the turbid state again.

By the same method as that mentioned above, the influence of hydrochloric acid, acetic acid and butyric acid on urease was examined.

TABLE III.

Concentration of acid	N/10 NH_3 developed in 20·5 hrs in cc.		
	HCl	Acetic acid	Butyric acid
N/1000	—	15·87	18·99
N/2000	16·09	24·06	23·81
N/2500	24·17	24·29	23·50
N/3330	24·12	24·28	23·78
N/5000	—	24·38	23·82
Control urease without acid	24·25	24·38	23·76

By these experiments it was proved that all acids employed at a strength of N/2500 did not affect urease.

If we want to compare the effect of more diluted acids on urease it is necessary to use precipitated urease instead of water extract of soy-bean, because the latter contains much regulatory substance, which may protect urease from being inhibited by diluted acid.

All the following experiments, unless there is some note otherwise, were carried out with a solution of precipitated urease. At first I endeavoured to obtain some idea of the relative strength of various acids against urease.

N/500 acids were allowed to act on 5 cc. of 0·5 % urease for 17 hours, when they were neutralised, mixed with urea and left at room temperature for 22·5 hours. The amount of NH_3 evolved from these mixtures is shown in Table IV.

The NH_3 developed was very small in amount, proving that even weak acids are able to destroy urease in process of time.

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In order to find out the length of time necessary for the action of acids on urease, N/500 acids were allowed to act on 5 cc. of 0·5 % urease for 15,

TABLE IV.

Name of acid	Developed N/10 NH_3 in cc.	
0·5 % urease alone	23·62	23·60
,, ,,	23·59	
HCl	0·18	0·19
,,	0·19	
Oxalic	0·22	0·22
,,	0·21	
Formic	0·22	0·21
,,	0·20	
H_2SO_4	0·33	0·28
,,	0·22	
H_3PO_4	0·33	0·27
,,	0·21	
Acetic	0·36	0·36
,,	0·35	
Butyric	0·41	0·37
,,	0·32	

30, 60 and 90 minutes, the acids were then neutralised, urea was added and the mixture left at room temperature for 16·5 hours. Urease action was then checked and NH_3 estimated.

TABLE V.

Acids neutralised after	Developed N/10 NH_3 in cc.	
	H_2SO_4	CH_3COOH
15 minutes	0·87	—
30 ,,	0·61	5·64
60 ,,	0·26	4·50
90 ,,	0·20	1·65
0·5 % urease alone	23·81	

We see here a very distinct difference between a strong and a weak acid in regard to the velocity of their inhibitory effect. Regarding these facts, I varied the length of time of exposure of urease to acids and the strength of acids employed. A number of experiments were carried out, the following acids being used: HCl, HNO_3, H_2SO_4, H_3PO_4, formic, acetic, butyric, oxalic. trichloracetic, benzoic and salicylic.

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TABLE VI.

N/1000 acids were allowed to act upon 5 cc. of 0·5 % urease for 10 minutes, then neutralised, mixed with urea and left at room temperature for 16 hours.

Name of acid	Developed N/10 NH_3 in cc.	
0·5 % urease alone	23·85 23·79	23·82
" "		
Trichloracetic	0·48 0·60	0·54
"		
Oxalic	0·38 0·73	0·56
"		
HCl	0·61 0·61	0·61
"		
Formic	2·40 2·17	2·29
"		
Butyric	23·69 23·24	23·47
"		
H_3PO_4	23·56 23·69	23·63
"		
Acetic	23·80 23·72	23·76
"		

TABLE VII.

N/1000 acids were allowed to act upon 5 cc. of 0·5 % urease for one hour, then neutralised, mixed with urea and left at room temperature.

Name of acid	Developed N/10 NH_3 in 22 hrs in cc.
H_3PO_4	3·06
Butyric	11·40
Acetic	15·02
	Developed N/10 NH_3 in 21 hrs in cc.
Salicylic	0·66
Benzoic	1·14
Acetic	15·19

TABLE VIII.

N/2000 acids were allowed to act upon 5 cc. of 0·5 % urease for one hour, then neutralised, mixed with urea and left at room temperature for 20·5 hours.

Name of acid	Developed N/10 NH_3 in cc.	
0·5 % urease alone		23·55
HNO_3	0·49 0·34	0·42
"		
Trichloracetic	0·48 0·38	0·43
"		
HCl	0·50 0·49	0·50
"		
H_2SO_4	0·65 1·01	0·83
"		
Salicylic		1·27
Formic		3·90
Benzoic		19·02

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TABLE IX.

N/3000 acids were allowed to act upon 5 cc. of 0·5 % urease for an hour, then neutralised, mixed with urea and left at room temperature for 18 hours.

Name of acid	Developed N/10 NH_3 in cc.	
0·5 % urease alone		23·87
Oxalic	21·47	20·83
,,	20·18	
Trichloracetic		21·15
HCl	23·15	23·26
,,	23·36	
HNO_3	23·37	23·50
,,	23·62	
H_2SO_4	23·58	23·62
,,	23·66	
Formic	23·92	23·76
,,	23·60	

TABLE X.

N/4000 acids were allowed to act upon 5 cc. of 0·5 % urease for an hour, then neutralised, mixed with urea and left at room temperature for 20·5 hours.

Name of acid	Developed N/10 NH_3 in cc.
0·5 % urease alone	24·35
Oxalic	21·22
H_2SO_4	23·57
HNO_3	23·63
HCl	23·72
Trichloracetic	23·77
Formic	23·78

If we arrange the acids according to their inhibitory effect upon urease, we get the following order.

The inhibitory effect decreases passing from left to right.

TABLE XI.

According to Table VI:
Trichloracetic, oxalic, hydrochloric > formic > butyric, phosphoric, acetic.
According to Table VII:
Salicylic > benzoic > phosphoric > butyric > acetic.
According to Table VIII:
Nitric, trichloracetic, hydrochloric > sulphuric > salicylic > formic > benzoic.
According to Table IX:
Oxalic > trichloracetic > hydrochloric, nitric, sulphuric, formic.
According to Table X:
Oxalic > sulphuric, nitric, hydrochloric, trichloracetic, formic.

If we arrange the above-mentioned acids according to the strength of their inhibitory effects on urease, we get a general order of acids from Table XI as follows:

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Oxalic > Trichloracetic > Hydrochloric, Nitric > Sulphuric > Salicylic > Formic > Benzoic > Phosphoric > Butyric > Acetic.

According to Ostwald, if one gram-molecule of each of these acids is dissolved in 1024 litres of distilled water, they are dissociated at 25°, as follows:

Trichloracetic acid	99·40 %
Salicylic acid	62·80 %
Formic acid	35·80 %
Benzoic acid	21·61 %
Acetic acid	12·61 %
Butyric acid	11·41 %
Oxalic acid	94·70 % (gram-molecule in 256 litres).

This order of hydrogen ion concentration of nearly N/1000 acids corresponds with the order of acids which I obtained in Tables VI, VII and VIII. But there is one important exception, namely, butyric acid (Tables VI and VII). This, in spite of a lower hydrogen ion concentration than acetic acid, had an inhibitory effect on urease greater than that of acetic acid. This fact corresponds precisely with the result of J. Loeb [1909], who found butyric acid to have a stronger influence on membrane construction of sea-urchin eggs even than mineral acids. He remarked that acids in their physiological action are not dependent only upon their hydrogen ion concentration, and he is of opinion that the undissociated part of an acid plays the main rôle concerning its relative permeability through the cell membrane of eggs, and that the process may be of a purely physical nature.

According to Ostwald the equivalent conductivity of N/1000 acids at 18° is as follows:

HCl	375 practically totally dissociated,
HNO_3	373,
$1/2H_2SO_4$	355,
$1/3H_3PO_4$	106,
CH_3COOH	41.

If we compare the order of acids obtained in this work with the degree of dissociation of acids given above, we see that the former coincides with the latter fairly well.

At a dilution of N/3000 and N/4000 it is considered that the strong mineral acids such as HCl, HNO_3 and H_2SO_4 are almost entirely dissociated. Organic

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acids with high dissociation coefficients, as trichloracetic acid and oxalic acid, will be in the same dilution also almost entirely dissociated. Yet I have confirmed, by Prideaux's colorimetric method, that they have a smaller hydrogen ion concentration than HCl in the same dilution. Notwithstanding, we find a markedly stronger inhibitory effect of trichloracetic and oxalic acid than mineral acids. These exceptional cases force us to take other considerations than the exclusive idea of hydrogen ion concentration into account.

It is true that the hydrogen ion concentration of acids plays the main rôle in many purely chemical reactions. According to Ostwald, the inversion order of acids is: HCl, HNO_3, H_2SO_4, tri-, di-, mono-chloracetic acid, formic acid, acetic acid. It is just the order of hydrogen ion concentration. On the other hand there are many contradictions with regard to the physiological actions of acids.

Paul, Birnstein and Reuss obtained the order of acids as follows, classified by their bactericidal action [see Traube, 1912]:

Trichloracetic acid, HNO_3, HCl, oxalic acid, H_2SO_4, H_3PO_4, formic acid, acetic acid, butyric acid.

Barratt [1904] found that univalent aliphatic acids are more toxic than mineral acids on *Paramoecium*. Fühner and Neubauer [1907] showed that the limiting concentration of acids capable of causing haemolysis does not correspond to their hydrogen ion concentration.

Pauli [1910] investigated the order of acids in regard to their influence on the internal friction and the precipitability of solutions of albumin by alcohol, and found it to be:

Trichloracetic acid, dichloracetic acid, H_2SO_4, HNO_3, HCl, monochloracetic acid, acetic acid.

He ascribed these effects not to the strength of acids, but to the nature of their anions.

Loeb's experiments on parthenogenesis have been mentioned already. Here again the order of hydrogen ion concentration is not the order of activity. As to the results of the present work it shows us that the effect of acids on urease is mainly dependent upon hydrogen ion concentration, but not upon negative ions, because the influence of salts of these acids is not apparently so strong, as my later experiments and van Slyke's "salt effect" show. But on the other hand my experiments show us that there are some exceptions to the order of hydrogen ion concentration, viz. butyric acid, trichloracetic acid and oxalic acid.

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By these facts we are forced to consider as important the properties of acid radical ions, or undissociated acids, which may act in the way of facilitating the contact of enzyme particles and hydrogen ions.

Among the physical properties of acids or acid radical ions the surface tension is perhaps the most important, assuming that enzyme action takes place at the surface of enzyme particles.

According to Traube's [1909] measurements the constant of surface tension (γ) of molecular solutions of acids is as follows. The constant $\frac{rhs}{2}$ is given in mg./mm. and was measured at 15°. The constant (γ) for water was taken as 7·30:

acids:	$\frac{1}{2}H_2SO_4$	HCl	HNO_3	formic	acetic	propionic	butyric	trichloracetic
γ:	7·285	7·23	7·19	6·84	6·05	4·82	3·31	4·82

It is known that the less the surface tension the greater is the partition coefficient and adsorption coefficient. In adsorption, the adsorbed substances can carry other molecules or ions with them. According to these considerations the inhibitory action of acids on urease is, I assume, a function of hydrogen ion concentration and of surface tension, which latter gives the ions more opportunity of combining with urease particles in the diluted condition. The greater the lowering of surface tension, the more will the relatively smaller hydrogen ion concentration of the weaker acids be compensated by having more opportunity to come into touch with the urease particles.

By means of these considerations the exceptional cases of butyric and trichloracetic acids are explained. Many results concerning the biological effects of acids on bacteria, haemolysis, paramoecium, colloidal precipitation and parthenogenesis will be found to receive a common explanation in this way.

There is still only one difficulty to explain, that is, the superior efficiency of oxalic acid. This acid is not one with a very strong surface-tension activity as is trichloracetic acid. Some other factors, probably physical ones, remain to be detected to explain this exception.

(2) *Effects of caustic soda and ammonia on urease.*

Takeuchi, Armstrong and Marshall reported the inhibitory effects of alkalis on urease. Van Slyke and Zacharias [1914, p. 208] allege that the hydroxyl ion retards *d* but facilitates *c*. In accordance with this the optimum of urease action in diluted urea solution is more on the alkaline side, because in the diluted urea solution the combination of urease and of urea is retarded.

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At first I experimented with neutralised 2 % watery extract of soy-bean. To 5 cc. of this extract caustic soda and urea were added, bringing the content of each bottle to equal volumes, the mixture left at room temperature for twenty hours, the urease action then stopped and ammonia estimated.

TABLE XII.

Concentration of NaOH	Developed N/10 NH_3 in cc.
2 % bean extract alone	19·93
N/10	0·18
N/50	0·26
N/100	1·47
N/500	0·95

We see here that caustic soda inhibited urease action even at a concentration of N/500.

By the same method an experiment was made with ammonia. From the quantity of ammonia obtained the known quantity of ammonia added was subtracted with the following result.

TABLE XIII.

Concentration of NH_3	Developed N/10 NH_3 in cc.
2 % bean extract alone	24·32
N/20	12·35
N/50	21·46
N/100	24·05
N/200	24·50

We see here that the inhibitory action of ammonia is much inferior to that of soda. N/50 ammonia had a slight depressing effect.

I have also examined the effect of soda on precipitated urease.

TABLE XIV.

NaOH was allowed to act upon 5 cc. of 0·5 % urease for 1·5 hours, then neutralised, mixed with urea and left at room temperature for 22 hours.

Concentration of NaOH	Developed N/10 NH_3 in cc.
0·5 % urease alone	22·85
N/50	0·16
N/100	8·60
N/250	18·67
N/500	21·00
N/1000	22·77

It was thus found that N/50 soda totally destroyed the urease in 1·5 hours, while N/500 soda had a slight action and N/1000 soda had no effect.

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If we compare the results obtained in Tables XIII and XIV we see that the inhibitory effect of N/500 soda and N/50 NH_3 are about equal. But soda was allowed to act on urease only 1·5 hours, while N/50 NH_3 was left for 20 hours with urease. Thus we can assume with certainty that the effect of N/500 soda must be greater than that of N/50 NH_3.

Marshall, and van Slyke and his co-workers, ascribed the depressing effect of alkalis and ammonia on urease to their hydroxyl ion concentration. But there is another possibility that the hydrolysis of urea by urease may be a reversible one.

According to Kohlrausch, the equivalent conductivity at 18° is nearly 204 for N/500 NaOH, only 3·3 for N/10 NH_3 and 9·6 for N/100 NH_3. It is evident that we cannot explain the effect of alkali and ammonia on urease only by hydroxyl ion concentration. The difference of surface tension of soda and ammonia is also not enough to afford the required explanation.

We may therefore suggest that the effect of soda may be the effect of hydroxyl ion concentration and the effect of ammonia be partly due to the reversible reaction of hydrolysis of urea by urease, but this latter possibility is very doubtful.

(3) *Effects of neutral salts and alkaloids on urease.*

As is seen in the previous experiments the depressing effect of ammonia was far below that of soda. We may expect therefore that if neutral salts of strong alkalis be added to the digestion bottle and their bases be displaced by ammonia, the more or less depressing effect on urease, according to the kind of alkali, can be seen.

Van Slyke and his coadjutors found that neutral salts retard *c* and, if their concentration exceeds a 2 M solution, also retard *d*. If my assumption is right, van Slyke should have obtained the result that neutral salts retard *d* and facilitate *c*, because in my opinion alkalis displaced by ammonia will have an influence on urease.

In order to investigate this matter the following experiments were made.

At first neutral salts were mixed with 5 cc. of 0·5 % urease and urea, and after standing at room temperature for 20 hours urease action was checked and ammonia was estimated. It was noticed that the mixture showed a fine flocculent precipitation directly after adding $CaCl_2$, $BaCl_2$, and $HgCl_2$ to urease.

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TABLE XV.

N/10 salt solution	Developed N/10 NH_3 in cc.	
0·5 % urease alone	22·73	22·78
" "	22·82	
NaCl	20·32	19·85
"	19·38	
$CaCl_2$	22·03	22·18
"	22·32	
$BaCl_2$	22·68	22·20
"	21·72	

As I supposed N/10 NaCl has an apparent depressing effect while N/10 $CaCl_2$ and N/10 $BaCl_2$, in spite of precipitation, showed only a slight depressing effect. We can understand the marked depressing effect of 2 M solutions of neutral salts on urease as observed by van Slyke.

In the salt solutions of the present experiment the number of chlorine ions are equal, therefore these different depressing effects of neutral salts must be ascribed to the nature of the metallic ions. If the action of metallic ions is a tendency to colloidal precipitation of urease, we should expect more effect of the multivalent ions as $Ba^{\cdot\cdot}$ and $Ca^{\cdot\cdot}$ than of the univalent $Na^{\cdot}$ ions. This was not the case.

It was concluded therefore that the metallic bases of these neutral salts were displaced by ammonia hydrolysed from urea, owing to the law of mass-action. Metallic bases are able to dissociate more hydroxyl ion than their equivalent of ammonia. As to the degree of dissociation, sodium stands of course far beyond barium and calcium.

Van Slyke reported that neutral salts disturb the combination of enzyme and substrate, so that, when the concentration of urea becomes smaller at the end of the reaction, the depressing effect of neutral salts on the combination of urea and enzyme becomes more manifest.

If the urease action is checked at an early stage, then one would see less effect of neutral salts. The data of Table XVI agree with this assumption.

TABLE XVI.

N/10 salt solution	Developed N/10 NH_3 in 17·5 hours in cc.
0·4 % urease alone	12·10
NaCl	5·73
$BaCl_2$	5·52
$CaCl_2$	5·62
$HgCl_2$	0·00

But we can explain this result otherwise, assuming that hydroxyl ion concentration is effective on urease only when it exceeds some limit.

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In an early period of the reaction the hydroxyl ion concentration would not perhaps reach the effective limit. That is the reason why we do not see in Table XVI a distinct difference between Na, Ba and Ca ions in their effects.

$HgCl_2$ totally destroyed urease, as Hata [1909] found in the case of various ferments; it is to be ascribed to the special nature of the mercuric ion. I did not try to restore the lost urease action by K_2S.

Necessarily we are forced to ask, What will be the result if we use salts of weaker alkaline substances than ammonia? We can expect with reason to see accelerating effects. I used for this purpose the salt of an alkaloid (quinine hydrochloride).

TABLE XVII.

H_2O in cc.	1 % quinine hydrochloride in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 17 hrs in cc.
40	—	5	5	10·65
35	5	,,	,,	13·25
37·5	2·5	,,	,,	11·74
39	1	,,	,,	11·49

After standing for 17 hours the mixture in the digestion bottles became turbid. It indicates that quinine was precipitated, being freed from its salt by the action of the ammonia developed.

We find in this experiment that 0·02–0·1 % quinine hydrochloride has a marked accelerating effect on urease, as I supposed. But if I continued this experiment, I should no longer find this effect, because the free quinine base is toxic for urease. Table XVIII shows us this fact. Quinine base is difficult to dissolve in water. Therefore 0·06 g. of quinine was dissolved at first in about 2 cc. of absolute alcohol and was diluted with distilled water to 100 cc. Such a small quantity of ethyl alcohol has no effect on urease as is shown later on.

TABLE XVIII.

H_2O in cc.	0·06 % quinine in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 22 hrs in cc.
40	—	5	5	6·84
—	40	,,	,,	2·87
20	20	,,	,,	4·01
30	10	,,	,,	4·65
35	5	,,	,,	5·01

It is evident that free quinine base inhibits urease action apparently even in a concentration of 0·006 %. This effect is not due to the hydroxyl ion

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because 0·006 % quinine is only about mol./6300; it is apparently the effect of the alkaloid ion.

Laqueur [1906] examined the effect of quinine hydrochloride on various ferments. In accordance with many other workers he observed that quinine hydrochloride, generally speaking, inhibited enzymes, but sometimes in its diluted concentration accelerated the ferment action. As indicated by Traube and the author [1914], and as the present work suggests, it is desirable to distinguish between the results obtained by quinine salt and quinine base. It would be wrong to ascribe the effect of "quinine salts" to an effect of "quinine," as is sometimes done.

The same inhibitory results were observed with free atropine and pilocarpine base. Mixing of atropine and pilocarpine had only an additive effect but not an antagonistic effect on urease. The experiments of this section show that the effects of neutral salts are due to the alkalinity of metal bases and alkaloid bases to a certain limit. They do not agree with van Slyke's results concerning *c* and *d*.

(4) *Effects of alcohols on urease.*

Armstrong, Marshall, and van Slyke reported the retarding effect of alcohols on urease. Armstrong showed that alcohols which have less solubility retard the urease action more strongly than alcohols with greater solubility. Van Slyke stated that ethyl alcohol in 30 volumes per cent. (5·7 mol.) retarded both *c* and *d*, especially the latter. He ascribed this effect to the high osmotic pressure of the alcohol solution. He stated that a 2 M solution of alcohol is the limiting concentration for retardation of *d* as well as of *c*.

Alcohols are substances which are able to lower the surface tension of water considerably and we may therefore expect here some considerable

TABLE XIX.

H_2O in cc.	5 M alcohol in cc.	0·5 % urease in cc.	1·5 % urea in cc.	Conc. of alc. in M	N/10 NH_3 developed in 5 hrs in cc.	
20	—	5	5	—	9·43	9·41
,,	—	,,	,,	—	9·39	
—	Methyl 20	,,	,,	3·3	8·75	8·84
—	,,	,,	,,	,,	8·93	
—	Ethyl 20	,,	,,	,,	7·65	7·30
—	,,	,,	,,	,,	6·95	
—	Propyl 20	,,	,,	,,	7·62	7·31
—	,,	,,	,,	,,	7·00	
—	M/8 amyl 20	,,	,,	1/12	8·80	8·78
—	,,	,,	,,	,,	8·76	
30	M/2 paraldehyde 20	,,	,,	1/6		1·15

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effects of strongly active substances, like alcohols, on urease. Can it be justifiable to take into consideration here only the osmotic pressure-capacity factor of osmosis of Traube?

My experiments were directed at first to confirm the results obtained by others. Concentrated alcohol solutions were employed (Table XIX).

Addition of paraldehyde caused immediate precipitation of urease solution.

We see from this table an obvious depressing effect of methyl, ethyl, and propyl alcohol in 3·3 M and amyl alcohol in M/12. Paraldehyde in M/6 inhibited urease considerably.

This order of alcohols in relation to their depressing effect is the order of decrease of solubility, surface-tension activity and "attraction pressure" of Traube. The reason why the difference of effect between ethyl and propyl alcohol is not so distinct as is expected from the surface-tension activity, may be the too high concentration of alcohols used, or the time during which the digestion bottles were left at room temperature which was not suitable for the observation of this difference (see later).

Experiments were next carried out to examine the effect of diluted alcohols. The results were contradictory to the reports of previous investigators. In order to attain certainty the experiments were repeated carefully.

TABLE XX *A*.

Concentration of alcohol	N/10 NH_3 developed in 18 hours in cc.
0·5 % urease alone	23·52
M methyl	23·63
M ethyl	23·87
M/10 amyl	23·87
M/16 amyl	23·83

TABLE XX *B*.

Concentration of alcohol	N/10 NH_3 developed in 7 hours in cc.
0·5 % urease alone	6·66
M methyl	7·69
M ethyl	7·98
M/10 amyl	8·09

TABLE XX *C*.

Concentration of alcohol	N/10 NH_3 developed in 6·7 hours in cc.	
0·5 % urease alone	7·25	7·25
,, ,,	7·24	
M methyl	8·86	8·92
,,	8·97	
M ethyl	8·75	8·65
,,	8·55	
M propyl	8·53	8·64
,,	8·75	
M/20 amyl	8·50	8·42
,,	8·34	

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Alcohols in diluted condition accelerate the urease action. It is beyond question; methyl, ethyl, and propyl alcohol in a concentration of 1 M and amyl alcohol in one of M/20 per litre.

Another experiment with more dilute alcohols was carried out.

TABLE XXI.

Concentration of alcohol	N/10 NH_3 developed in 7 hours in cc.	
0·5 % urease alone	6·00	5·99
,, ,,	5·97	
M/5 methyl	5·97	6·08
,,	6·18	
M/5 ethyl		6·06
M/5 propyl	5·90	6·21
,,	6·51	
M/40 amyl	6·47	6·46
,,	6·44	

It is evident that methyl, ethyl and propyl alcohol in M/5 had only a feeble accelerating effect, but M/40 amyl alcohol still had a somewhat more recognisable accelerating action.

It is necessary to notice here that, in order to find the accelerating or retarding effect of some substance on ferments, it is very important that the conditions of duration of ferment action and relative concentration between ferment and substrate must be carefully adjusted.

In Table XX *A* we cannot see distinctly the accelerating effect of alcohol. The reason for this is the longer duration of the digestion, so that the process of hydrolysis went on nearly to the end, both with and without the addition of alcohols. On the other hand, to confirm the above assumption, I have experimented with relatively concentrated alcohol solutions. In this case the concentration of alcohol was the same as in Table XX—1 M, but the urease solution was relatively less concentrated, 0·4 % being used instead of 0·5 %. The digestion bottles were also left a little longer at room temperature.

TABLE XXII.

Concentration of alcohol per litre	N/10 NH_3 developed in 22 hours in cc.	
0·4 % urease alone	22·99	22·90
,, ,,	22·80	
M methyl	22·78	22·72
,,	22·66	
M ethyl	22·63	22·67
,,	22·71	
M propyl		22·72
M butyl	22·79	22·77
,,	22·76	
M/40 amyl	22·80	22·80
,,	22·80	

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As is seen, there was a slight depressing effect produced by 1 M solutions of the alcohols on urease. That is quite contradictory to the result where this same concentration of the alcohols was able to accelerate the urease action.

Provided that the conditions mentioned above are fulfilled, we learn that concentrated alcohols inhibit urease, while diluted alcohols, on the contrary, accelerate its action.

It is probable that many previous investigators did not succeed in observing this accelerating effect of alcohol on the urease action, because the conditions mentioned above were not satisfied. The alcohol solutions may have been absolutely or relatively too concentrated or the duration of urease action too short.

There are still some points of interest to be considered. It is strange that here alcohols facilitate the enzyme action, while we are taught that alcohols usually paralyse various chemical and biological reactions. My result however is not the only one of the kind, since Giselt [1905] has observed the accelerating effect of alcohol on the action of pancreatic lipase.

Now again, the question arises: To what properties of alcohols are their inhibitory and accelerating effects on urease due?

If we try to explain these facts only by osmotic pressure, then it is rather curious that one property can act in two contrary directions.

Traube alleged long since that osmotic pressure is only a capacity factor of the mechanics of osmosis—number of the dissolved particles—and another important driving factor of osmosis—intensity factor—is neglected. Traube called the latter attraction pressure or "Haftdruck." The substance which has less attraction pressure against water accumulates on the surface of the second phase in contact with water, and lowers the surface tension of water. This fact has long been known as Gibbs' theorem and has been experimentally proved.

We do not therefore exhaust all the possibilities when we take into consideration only the osmotic pressure in dealing with substances which have a large effect on surface-tension and with colloidal particles having immense surface development.

With regard to the cause of the accelerating effect of dilute alcohols on urease action there are two possibilities in reference to the considerations mentioned above.

1. Alcohol molecules carry urea molecules with them together to the surface of urease particles, when alcohol molecules accumulate on the surface of enzyme. This process facilitates the contact between enzyme and substrate.

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2. Change of surface-tension of urease solution disperses the urease particles and thus increases their active surface.

With regard to the depressing effect of concentrated alcohols on urease there are also two possible causes.

1. Alcohol molecules accumulating on the surface of enzyme particles disturb the contact between urease particles and urea molecules or drive off urea molecules from the surface of enzyme particles.

2. Enormous change of surface-tension favours aggregation of enzyme particles.

Supposing that the first consideration might be the case, I planned my experiment as below.

We learned that formic acid had a moderate inhibitory effect on urease. If we add urease to a mixture of alcohol and formic acid we may expect the inhibitory effect of formic acid to be increased by dilute alcohol, and we may expect with the concentrated alcohol, on the contrary, the diminution of the inhibitory effect of formic acid.

Formic acid was mixed and shaken with ethyl alcohol, and water and 0·5 % urease were then added. In the whole mixture the formic acid was N/2000 and alcohol was 1 and 3·3 molar respectively. The acid was allowed to act on the urease for 15 minutes, and was then neutralised. Urea was added, each bottle was brought to an equal quantity of content and left for 22 hours at room temperature.

TABLE XXIII.

H_2O in cc.	N/200 formic acid in cc.	5 M ethyl alcohol in cc.	0·5 % urease in cc.	Concentration of alcohol M		M/200 NaOH in cc.	1·5 % urea in cc.	H_2O in cc.	N/10 NH_3 developed in 22 hrs in cc.	
40	—	—	5	—		—	5	5		23·18
35	5	—	,,	—	neutralised by addition of	5	,,	—	10·35	10·6
,,	,,	—	,,	—		,,	,,	—	9·76	
25	,,	10	,,	1		,,	,,	—	10·14	10·15
,,	,,	,,	,,	,,		,,	,,	—	10·16	
2	,,	33	,,	3·3		,,	,,	—	13·08	12·54
,,	,,	,,	,,	,,		,,	,,	—	11·99	

The bottles with 1 M ethyl alcohol and acid developed practically as much ammonia as the bottles with acid alone. But if we consider that 1 M ethyl alcohol is able to accelerate the urease action we come necessarily to the conclusion that 1 M ethyl alcohol facilitated the inhibitory effect of formic acid. In the case of 3·3 M ethyl alcohol we may expect the additional result of the inhibitory effect of acid and the depressing effect of concentrated alcohol if we do not take into consideration the influence of the change of

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surface-tension. In fact, we find in this experiment a distinct increase of ammonia in the bottles with acid and 3·3 M ethyl alcohol. It shows us that the action of the urease particles has been relieved from acid inhibition by concentrated alcohol. It has over-compensated the loss caused by the depressing effect of concentrated alcohol.

Let us consider the second possibility mentioned above. If the effect of 1 M alcohol was merely in the dispersion of enzyme particles, but not in facilitating the contact between substrate and urease particles, we must have a larger yield of ammonia from the bottles with acid and 1 M ethyl alcohol than from the bottles with acid alone. But this was not the case.

For the same reason, if 3·3 M alcohol had no effect in driving off acid from the surface of urease particles, but only the aggregating effect on urease, we must get a smaller yield of ammonia from the bottles with 3·3 M ethyl alcohol and acid than from the bottles with acid alone. But this was also not the case.

The question next arises, Does not alcohol combine with formic acid to deprive the latter of its inhibitory effect?

As compared with the concentration of N/2000 formic acid, 1 and 3·3 M ethyl alcohol are very concentrated. If the process concerned is only ester formation between alcohol and formic acid, then 1 and 3·3 M alcohol are strong enough to combine with all the formic acid. We may expect then in Table XXIII only the accelerating effect of diluted alcohol and the retarding effect of concentrated alcohol. But this also was not the case, the result was the reverse.

Further, if the effect of dilute alcohol is in the facilitation of combination between urease and urea, and the effect of concentrated alcohol is in the driving off urea from the surface of urease particles, we can assume as follows. If concentrated urea solution is used as substrate, then the combination of urea molecules with urease particles will be so easy that addition of 1 M alcohol has no effect. On the other hand in such conditions urease particles will be surrounded by so many urea molecules that addition of concentrated alcohol will not be able to drive off the urea molecules from the surface of urease particles in order to take their place.

For this purpose I used 5 % urea solution instead of the usual 0·15 %.

This result proves the above assumption to be true. 3·3 M ethyl alcohol even accelerated the urease action a little. Compared with the high concentration of urea, 3·3 M ethyl alcohol is a relatively diluted solution, so the driving power of alcohol is not stronger than the adhering power of

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TABLE XXIV.

H_2O in cc.	5 M ethyl alcohol in cc.	0·5 % urease in cc.	25 % urea in cc.	Concentration of alcohol M	N/10 NH_3 developed in 7 hours in cc.	
35	—	5	10	—	7·14	7·20
,,	—	,,	,,	—	7·25	
25	10	,,	,,	1	7·60	7·40
,,	,,	,,	,,	,,	7·19	
2	33	,,	,,	3·3	7·53	7·72
,,	,,	,,	,,	,,	7·90	

urea to urease. This must be the reason of the acceleration. 1 M ethyl alcohol accelerated only a little. These results teach us also that the effect of alcohol is not in a dispersion or aggregation of urease particles.

There is still one more question. According to Traube the lowering of surface-tension by methyl, ethyl, propyl, butyl and amyl alcohol increases in the proportion of $1:3:3^2:3^3:3^4$. But in my series of experiments I could not see such enormous differences in the effect of these alcohols. I am of opinion that this was due entirely to unsatisfactory experimental conditions. I tried, therefore, to resolve this question from the other side. I prepared isocapillary alcohol solutions and examined their effect on urease.

Traube's stalagmometer was used for this purpose. It contained 56·9 drops of water at 20°; 5 M methyl alcohol showed 79·1 drops. The number of drops of alcohol solutions and the data of the experiments are as follows. The dilution of alcohols to make isocapillary solutions corresponded nearly to the law of Traube mentioned above.

TABLE XXV.

H_2O in cc.	10 cc. of alcohols: Name	10 cc. of alcohols: No. of drops water	1·5 % urea in cc.	0·5 % urease in cc.	N/10 NH_3 developed in 6·7 hours in cc.	
40	—	56·9	5	5	14·30	14·23
,,	—	,,	,,	,,	14·16	
30	Methyl	79·1	,,	,,	15·46	15·48
,,	,,	,,	,,	,,	15·50	
,,	Ethyl	79·0	,,	,,	14·74	15·09
,,	,,	,,	,,	,,	15·44	
,,	Propyl	78·9	,,	,,	14·62	14·45
,,	,,	,,	,,	,,	14·28	
,,	Butyl	79·0	,,	,,	14·68	14·78
,,	,,	,,	,,	,,	14·87	
,,	Amyl	78·9	,,	,,	14·61	14·67
,,	,,	,,	,,	,,	14·72	

The results were quite decisive. All the alcohols employed accelerated nearly in the same degree. The alcohol solutions were not quite isocapillary but the number of drops of these solutions varied between 78·9 and 79·1.

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The results obtained were very instructive, because the yield of ammonia was exactly proportional to the number of drops, i.e. to the surface-tension. A difference of 0·1 drop in nearly 80 drops showed itself distinctly in the effect on urease action.

It is necessary to emphasise once more the fact that, in the discussion of the effect of substances, which have a large effect on surface-tension, on ferments, warning must be given earnestly against taking only osmotic pressure into consideration; another important factor, surface-tension, must be remembered. The present series of experiments proved this idea to be quite justified.

(5) *On the diminution of action of urease during preservation.*

Traube and the author [1914] proved by means of dark ground illumination that the diminution of toxicity of atropine in a watery solution during preservation is to be ascribed to the aggregation of colloidal particles of atropine. We supposed that this process would follow the formula of unimolecular reactions and in fact we found the (k) of this process fairly constant.

Van Eck [1911] found that the destruction of peroxidase in milk by heat proceeds as a reaction of the first order and the (k), obtained by using the formula of a unimolecular reaction, was constant, and this was confirmed by Zilva [1914].

It is not without interest, therefore, to investigate urease in this direction.

1 % urease solution was kept under toluene in a room where the change of temperature was not great. The bottle with urease was exposed to diffuse light. During 52 days' preservation the efficiency of the urease was estimated at intervals.

5 cc. of the urease solution were mixed with 5 cc. of 1·5 % urea and brought up to 50 cc. with distilled water. The digestion bottle was kept in the water bath at 16° for 7 hours; then the urease action was checked and ammonia was estimated.

During preservation some amount of precipitation took place.

In order to find out the relation between the concentration of urease and the development of ammonia by urease I performed the following experiment.

At the start of this experiment out of the above-mentioned 1 % urease solution 5, 3·5, 2·5, 1·67, 1·25 and 1·0 cc. had been measured and the amount of NH_3 developed by these quantities of urease under the same conditions as stated above was determined. The relation of urease concentration and yield of ammonia was the following:

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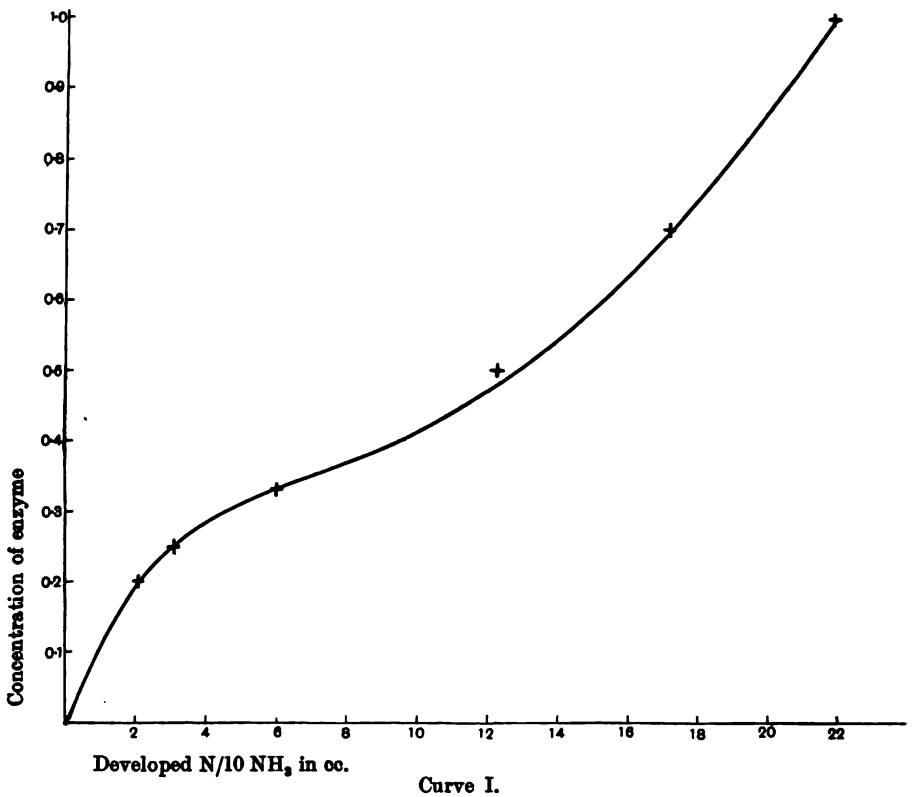
TABLE XXVI.

Concentration of enzyme	N/10 NH_3 developed in cc.
1·0 (5 cc. of 1 % urease)	21·65
0·7	17·17
0·5	12·20
0·33	5·96
0·25	3·01
0·2	2·02

These data are shown graphically in Curve I.



Curve I.

The following curve and table show the amount of ammonia developed by preserved urease and the calculated concentration of urease on the various days of experiment. This calculation was carried out by making use of Curve I and the quantity of developed ammonia.

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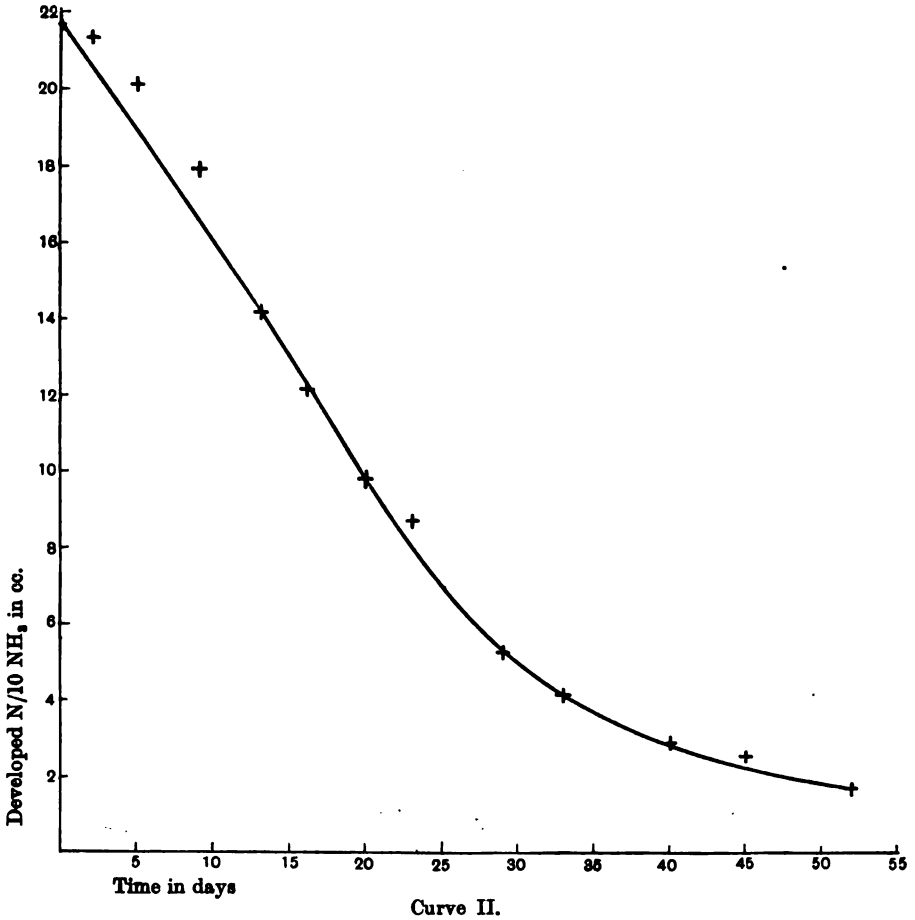

Curve II.

TABLE XXVII.

Days of preservation	N/10 NH_3 developed in cc.	Calculated concentration of urease		k
$t=0$	21·65	1·00 = a		
2	21·37	0·97	$a-x$	0·0152
5	20·10	0·88		0·0256
9	17·96	0·74		0·0334
13	14·16	0·55		0·0460
16	12·16	0·48		0·0459
20	9·80	0·41		0·0446
23	8·71	0·38		0·0421
29	5·26	0·32		0·0465
33	4·10	0·29		0·0375
40	2·90	0·25		0·0346
44	2·55	0·23		0·0333
52	1·72	0·17		0·0341

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If a unimolecular reaction is not reversible we have the well-known formula:

$$k = \frac{1}{t} 2{\cdot}302 \log \frac{a}{a-x}.$$

I took here 24 hours as a unit of t and obtained k as shown above.

A steady change of k was here observed; it reached its maximum on the 29th day and then began to fall.

Summary.

1. The effect of various acids, alkalis, alcohols, aldehyde, neutral salts and alkaloids on the urease of soy-bean has been studied.

2. In the inhibitory effects of inorganic and organic acids on urease the hydrogen ion concentration plays the main rôle. These inhibitory effects are influenced by the physical properties, and especially the surface-tension, which the acids possess. Total inhibitory effects are considered to be the combined effects of chemical and physical properties. If the dissociation of the acids is equally strong, then the inhibitory effects of acids with greater effect on surface-tension obtain the upper hand. Even the deficiency of hydrogen ion concentration can be over-compensated by great effect on surface-tension. The acids with great effect on surface-tension facilitate the adsorption of hydrogen ions on the surface of urease particles.

3. The inhibitory effects of caustic soda and ammonia do not coincide in reference to the hydroxyl ion concentration. Soda inhibited urease in far lower concentration than ammonia. But in equally effective soda and ammonia solutions the hydroxyl ion concentration is far greater in soda solution. The inhibitory effect of soda is to be ascribed to the hydroxyl ion concentration but ammonia has some further action.

4. Provided that certain conditions are fulfilled, it has been proved that 1 M alcohols (methyl, ethyl, propyl) and M/40 amyl alcohol accelerate the urease action, but 3·3 M of the former group and M/12 of the latter retard the urease action.

5. Isocapillary alcohol solutions manifest equal effects on urease.

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6. The cause of this acceleration or retardation is not to be understood merely in the light of osmotic pressure—Traube's capacity factor of the mechanism of osmosis. The intervention of Traube's intensity factor—attraction pressure, represented here by the surface-tension—makes clear the explanation of these phenomena.

7. The dilute alcohols which lower the surface-tension of the solvent accumulate on the surface of the second phase in contact with the solvent. By this action they facilitate adsorption between urease and urea. This is probably the cause of the acceleration of the urease action. Concentrated alcohols disturb the adsorption between urease and urea by displacement. This is the cause of retardation of the urease action.

8. In this work it is not found that the effects of alcohols on urease were caused by the dispersion or aggregation of urease particles.

9. Aldehyde inhibits urease notably.

10. The effects of neutral salts are merely those of their metallic bases. They retard in N/10 strength the urease action, because metallic bases displaced by ammonia produce a greater hydroxyl ion concentration than the equivalent of ammonia.

11. In accordance with this, alkaloid salts (quinine is a weaker base than ammonia) accelerate urease action in the first stage of hydrolysis. But alkaloid bases themselves inhibit urease action markedly (quinine, atropine and pilocarpine). The effects of the atropine and the pilocarpine on the urease are additive but not antagonistic.

In conclusion, I have pleasure in expressing my thanks to Prof. Starling, Prof. Bayliss and Dr Plimmer, for their kindness in allowing me to work in their institute and for their advice. The theme of this work was suggested by Prof. Bayliss and I worked under his supervision.

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LI. ON THE UREASE OF THE SOY-BEAN AND ITS "CO-ENZYME."

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In 1897 Bertrand introduced the term "co-enzyme." Since that time, co-enzymes have been discovered for various enzymes. The mode of action of co-enzymes is not yet quite clear, but the constitution of some co-enzymes is definitely determined. For instance, hydrogen ion for pepsin, hydroxyl ion for trypsin, electronegative ions such as chlorine and bromine ions for pancreatic amylase (Henri) and bile-salts for the lipase of liver (Magnus); [for literature see Bayliss, 1914, p. 123].

The constitution of other co-enzymes is not yet known and it is likely that these co-enzymes consist of a complicated system of several components. Harden and Young's [1906, 1908, 1910] co-enzyme of the zymase of yeast juice consists apparently of unknown components. Recently Neuberg and Schwenk [1915] found that salts of 2-ketonic acids act on yeast juice or dried yeast like the co-enzyme, but some important factor is wanting since the effect of salts of 2-ketonic acids is not as strong as that of the natural co-enzyme.

The "co-enzyme" of urease belongs also to the group of those with unknown constitution. Indeed, its nature, as will be seen, may throw some doubt on its claim to the title.

Experimental.

Methods employed.

The method employed for the estimation of ammonia which was produced by urease action was the same as that described in the previous paper [Onodera, 1915]. Only a few words may be added. Precipitated urease prepared according to van Slyke was used as the source of enzyme. The contents of each digestion bottle were always 50 cc. and that always included

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5 cc. of 1·5 % urea solution. This amount of urea corresponds to 25 cc. of N/10 NH_3. The digestion bottles were left in a water bath at room temperature.

1. *Urease loses its activity by dialysis.*

Dialysis was always carried out at room temperature by means of parchment paper, the urease solution being covered with toluene. Distilled water was used as a dialysate and it was either changed at intervals or continuously by means of a syphon. Dialysis was stopped when the chloride reaction of the dialysate was negative. The loss of activity of the enzyme by dialysis is due either to change of enzyme sustained during the course of dialysis or to the passing of enzyme particles through the parchment paper or to the loss of co-enzyme.

Loss of activity of enzyme owing to preservation is excluded since the control undialysed urease preserved under toluene was always very active (see below).

Urease is capable of passing to some extent through thin parchment paper, but not through the parchment paper employed in these experiments. Moreover an experiment is recorded in section 4 showing that this did not take place. It appears, therefore, that some essential constituent was removed from the urease by dialysis. This constituent may, for brevity, be called "co-enzyme."

In the following sections, several experiments are described proving that assumption.

2. *A small amount of fresh urease is able to revive the lost activity of the dialysed urease.*

TABLE I.

1·5 % urease dialysed for 5 days in cc.	0·5 % fresh urease added in cc.	N/10 NH_3 developed in 16·5 hours in cc.
5	—	0·08
5	0·5	4·62
5	1·0	8·53
—	1·5	4·03
—	1·0	1·23
—	0·5	0·15
Control urease not dialysed, 3 times diluted		
5	—	23·19

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As is seen in this table, 1·5 % urease lost its activity by 5 days' dialysis, and 5 cc. of this dialysed urease developed only 0·08 cc. of N/10 NH_3, while 5 cc. of the control urease, even three times diluted, hydrolysed almost all the urea present in nearly the same time. The addition of 0·5 and 1·0 cc. of 0·5 % fresh urease revived the lost action markedly, while the sum of the ammonia produced by the dialysed and the fresh urease each separately was much less.

The question arises whether this reviving action of the fresh urease is not an illusory phenomenon and whether it is not a simple additional effect of fresh urease on the urease which might be diluted by dialysis. The curve of urease concentration and ammonia development given in the previous paper [Onodera, 1915, p. 570] shows that the amount of ammonia produced by the quantity $(a + b)$ cc. of urease is greater than the sum of ammonia which is produced by a cc. and b cc. of urease respectively. From this curve we obtained the following data:

0·5 % urease in cc.	N/10 NH_3 developed in cc.
0·5	1·0
1·0	2·0
1·5	4·6
2·0	9·4
2·5	12·8
3·0	15·2
3·5	17·2
4·0	18·8

Making use of these data, if we suppose that a certain quantity of the dialysed urease can produce 1·0 cc. N/10 NH_3, then that quantity corresponds exactly with 0·5 cc. of 0·5 % urease. Now suppose that we add to that dialysed urease 1 cc. and 1·5 cc. of 0·5 % fresh urease respectively. By the simple addition of the corresponding quantities of ammonia we get 3 cc. and 5·6 cc. of N/10 NH_3 respectively. But in fact 1·5 and 2·0 cc. of 0·5 % fresh urease produced 4·6 and 9·4 cc. respectively.

The increase of the amount of ammonia produced by the further addition of urease, as is seen in the previous data, is not so great as it is in Table I.

In the former case, the increase of yield of ammonia does not exceed twice the arithmetic sum of the separate amounts of ammonia. In the latter case, the increase reaches to ten or twenty fold. According to these considerations, the presence of co-enzyme in the fresh urease and the loss of co-enzyme by dialysis was definitely settled.

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3. *Is this co-enzyme an inorganic salt and is it soluble in ether?*

A certain volume of 1 % urease was dried, incinerated and was dissolved in a quantity of distilled water equal to its original volume. 5 cc. of 1·5 % urease, which was dialysed for 5 days, developed 0·13 cc. of N/10 NH_3 in 16·5 hours. The same quantity of the dialysed urease with 5 cc. of the solution of the ash developed only 0·22 cc. of N/10 NH_3 during the same time.

Rosenheim's co-enzyme or activator of pancreatic lipase could be extracted from the ash only by means of hydrochloric acid [Pekelharing, 1912]. I tried this procedure also with urease. Urease was incinerated with hydrochloric acid and the ash was dissolved in water. 5 cc. of the same dialysed urease developed 0·33 cc. of N/10 NH_3 in 20 hours while an equal quantity of the dialysed urease with the solution of the ash produced only 0·36 cc. of N/10 NH_3.

20 cc. of 1 % urease were shaken with 50 cc. of ether and the ether separated. After evaporation of the ether the residue was suspended in 20 cc. of distilled water. 15 cc. of this solution mixed with 5 cc. of the dialysed urease developed 0·33 cc. of N/10 NH_3 in 20 hours, while the dialysed urease alone produced 0·36 cc. of N/10 NH_3 during the same time. I tried to ascertain whether the co-enzyme was in the original solution. The urease solution after ether extraction was freed from ether by drawing an air current through it, the aeration being continued nearly half an hour until the smell of ether disappeared. With this solution the following experiment was made.

TABLE II.

Dialysed urease in cc.	1 % urease after extraction with ether	N/10 NH_3 developed in 17·7 hours in cc.
5	—	0·67
—	5	1·84
5	5	2·62

It will be seen that the urease action was inhibited very strongly and no activator was found in it. We can judge from this fact that ether destroyed the co-enzyme and that it could not be restored by the driving away of ether.

The dialysed urease employed in this series of experiments was, of course, reactivated when fresh urease was added.

We can conclude that the co-enzyme is not an inorganic salt and does not pass into ether but is destroyed by ether.

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4. *Can the co-enzyme be found in the dialysate?*

For the purpose of solving this question, the following experiment was carried out. 0·5 % urease was subjected to dialysis for 4 days, the amount of dialysate being 5 times as great as the urease. The dialysate was not changed. After dialysis the dialysed urease solution was found in the same quantity as at starting. During dialysis both the urease solution and the dialysate were covered by toluene. The data obtained with these materials are given in Table III.

TABLE III.

Urease in cc.		N/10 NH_3 developed in 16·3 hours in cc.
0·5 % urease not dialysed	5·0	15·70
0·5 % urease dialysed	5·0	0·11
Dialysate	25·0	− 0·06
0·5 % urease dialysed Dialysate	5·0 25·0	0·22

By this experiment it was proved that the urease inactivated by dialysis could not be reactivated by the addition of dialysate. It was moreover shown that no urease particles passed through the parchment paper. This dialysed urease solution recovered its activity considerably when co-enzyme was added, as the following table shows.

TABLE IV.

Dialysed solution in cc.		0·5 % fresh urease in cc.	N/10 NH_3 developed in 16·5 hours in cc.
0·5 % urease not dialysed, as control	2·5	—	3·36
0·5 % urease dialysed ...	2·5	—	0·08
Dialysate	12·5		
0·5 % urease dialysed ...	2·5	1·0	7·70
Dialysate	12·5		
—		1·0	0·60

Co-enzyme was lost by dialysis but it could not be detected in the dialysate. What change then might have occurred to the urease during dialysis?

(*a*) Is co-enzyme dialysable but destroyed immediately after passing through parchment paper into the dialysate?

(*b*) Is co-enzyme not dialysable but changed into an inactive state through the diminution of salts owing to dialysis? It is possible that the precipitation of proteins may have been the principal cause. In fact, notable precipitation occurred in the urease solution during dialysis, while the control urease solution remained unchanged.

(*c*) Is co-enzyme adsorbed by the dialysing parchment paper?

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No definite ground could be found for answering the first question with confidence. It is however very unlikely.

To answer the second question, the following experiments were carried out. After dialysis, the dialysed urease solution was filtered and the precipitate was suspended in distilled water, the volume of the suspension being made equal to that of the filtered urease. (To avoid confusion I use in the following description the abbreviations (d.f.) for the filtrate and (d.p.) for the suspension of the precipitate from the dialysed urease.) With d.f. and d.p. experiments were carried out.

TABLE V.

1·5 % urease dialysed for 5 days in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 17 hours in cc.
d.f. 5	—	0·95
d.p. 5	—	0·88
d.f. 5 / d.p. 5	—	3·07
d.f. 5	2·0	16·05
d.p. 5	2·0	8·70
—	2·0	2·00
1·5 % urease not dialysed, as control 5	—	22·66

TABLE VI.

0·5 % urease dialysed in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 17 hours in cc.
d.f. 5	—	0·01
d.p. 5	—	0·23
d.f. 5	2·0	7·52
d.p. 5	2·0	5·62
—	2·0	1·70

In Table V we see that d.f. and d.p. were not able to activate each other. It indicates that co-enzyme was neither in the solution nor in the precipitate of the dialysed urease.

If we compare the activities of d.f. and d.p. alone, we do not find any distinct difference in Table V but in Table VI d.p. was a little stronger than d.f. When d.f. and d.p. had been activated by addition of fresh urease they showed a distinct difference. The activated d.f. developed markedly more ammonia than the activated d.p. The increased development of ammonia in the activated d.p. gives us no proof that the increase was chiefly to be ascribed to the activating effect of co-enzyme added (fresh urease).

From the data of Tables V and VI we may conclude that inactivated enzyme was present in the filtrate d.f. The precipitate d.p. contained a

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smaller quantity of enzyme proper than the filtrate, but still, although very small, some quantity of co-enzyme. This co-enzyme might possibly be supposed to be fixed by the enzyme. For this reason, the precipitate, in spite of its activity, could not activate the filtrate. From these facts I distinguish two kinds of co-enzyme of urease. One is the fixed co-enzyme and the other is the free co-enzyme.

In the following experiments it is clearly seen that the free co-enzyme has less resistance to various modes of treatment (such as dialysis, influence of heat, acid and alkali) than the fixed one. This fact agreed also with an experiment on the germination of soy-bean (see later).

Some reactions of the precipitate were studied. 1·5 % urease was subjected to dialysis for 4 days. The precipitate was collected and was made up with distilled water to a suspension equal in volume to the original solution. The dialysis was not quite sufficient, consequently the dialysed urease still showed some activity. The suspension was like milk and quite neutral. The addition of two drops of saturated $MgSO_4$ solution, 0·2 cc. of N/10 NaOH, or four drops of N/10 H_2SO_4 to 2 cc. of the suspension cleared away the milky state. This reaction was interesting, remembering that urease solution always becomes turbid when it is mixed even with a very weak acid. $CaCl_2$ had no effect. The suspension showed a distinct biuret-reaction. An experiment on digestion of the precipitate was made. 5 cc. of 1 % trypsin were added to 20 cc. of the suspension. The mixture was subjected to digestion at 37° for 2 days and was then dialysed for 2 days; the biuret-reaction was negative in this dialysed mixture.

An experiment was then made with the mixture.

10 cc. of the digested and then dialysed suspension developed 4·75 cc. of N/10 NH_3 in 18 hours.

5 cc. of 1 % trypsin developed 0·27 cc. of N/10 NH_3.

10 cc. of the suspension alone developed 7·42 cc. of N/10 NH_3 in 21 hours.

From this we learn that the urease was not destroyed by trypsin digestion. Van Slyke also found that urease resisted trypsin digestion.

I tried also to restore the co-enzyme from the precipitate, supposing this precipitation to be reversible. The precipitate was dissolved in a small quantity of NaOH, H_2SO_4, or $MgSO_4$ as described above and was added to dialysed urease, but these experiments all failed to restore the co-enzyme.

It seems very likely that the disappearance of co-enzyme was due to precipitation during dialysis and that it remained in the dialysed solution in an inactive state, but I could not succeed in proving it.

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As to the third question, the adsorption of co-enzyme by parchment paper was definitely excluded. The following experiment shows this.

5 cc. of dialysed urease developed 0·08 cc. of N/10 NH_3 in 16·4 hours.

5 cc. of dialysed urease with parchment paper cut in pieces developed 0·06 cc. of N/10 NH_3 in 16·4 hours.

5 cc. of dialysed urease + 1·0 cc. of 0·5 % fresh urease developed 8·53 cc. of N/10 NH_3 in 16·4 hours.

5. *Can co-enzyme be filtered through a Berkefeld porous filter?*

Rosenheim's co-enzyme of pancreatic lipase could easily be separated by filtration through Pukall's porous clay filter [Umeda, 1915]. The same experiment was therefore tried with urease.

After filtration, a white precipitate remained on the filter. The precipitate was collected and treated with a quantity of distilled water equal to that of the filtrate so as to make a suspension.

Investigations of the activity of the filtrate as well as of the suspension and of their contents of co-enzyme were carried out. The results obtained were as follows:

TABLE VII.

0·5 % urease dialysed in cc.	Urease treated with clay filter in cc.		N/10 NH_3 developed in 17·25 hours in cc.
	Filtrate	Residue	
5	—	—	0·13
5	2	—	2·48
5	—	2	3·92
—	2	—	0·68
—	—	2	0·80

TABLE VIII.

0·5 % urease dialysed in cc.	Urease treated with clay filter in cc.		N/10 NH_3 developed in 18·7 hours in cc.
	Filtrate	Residue	
5	—	—	0·08
5	1	—	3·82
5	—	1	1·27
—	1	—	0·62
—	—	1	0·09
—	5	5	22·07

As is seen in Tables VII and VIII, the activities of the filtrate and the residue were not constant; in the case of Table VIII the activity of the residue was very small. This difference was caused probably by the mode of preparing the urease solution and the conditions at filtration, but it was shown that

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no urease was lost by filtration through a porous filter. As Table VIII shows, the mixture of filtrate and residue in corresponding proportions had a very strong activity.

Further, the filtrate and the residue both contained co-enzyme. Their activity went quite parallel to their contents of co-enzyme. It was therefore clear that in the case of urease the enzyme proper and the co-enzyme both passed through the porous filter.

6. *Investigation of the distribution of urease in soy-bean or in its germ.*

It is known that some enzymes are distributed only in certain parts of plants, cells, etc. The distribution of the enzyme itself and of its co-enzyme might be different, so that the following experiment was made.

I separated the shell of the soy-bean from the bean substance or kernel and made a 1 % water extract from each of these two parts, with which experiments were made.

TABLE IX.

1·5 % urease dialysed in cc.	1 % extract of bean-shells in cc.	1 % extract of bean-kernel in cc.	N/10 NH_3 developed in 16·4 hours in cc.
5	—	—	0·45
5	2	—	0·50
5	—	2	1·73
—	2	—	−0·01
—	—	2	0·22

We learn from these data that the shell contained neither urease nor co-enzyme while the bean substance contained both.

I investigated then whether germination of the soy-bean was able to change the distribution of enzyme and co-enzyme.

Soy-beans were washed with distilled water and were arranged in rows on sheets of filter paper and were then covered again by several sheets of filter paper. They were all kept in glass vessels and moistened with distilled water. After keeping them in darkness for three days, the commencement of the growth of the germs was noticed. Here the germs, shell and proper substance were separated from each other. 1 % water extracts were prepared from each of the three parts and the following experiment made:

TABLE X.

5 cc. of 1 %	germ-extract	developed	17·34	cc. of N/10 NH_3	in 18·2	hrs.
,,	kernel-extract	,,	9·42	,,	,,	,,
,,	shell-extract	,,	0·18	,,	,,	,,

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The weights of dry substance of the germs and the bean substance proper were 10·47 and 38·79 % respectively. Consequently, a 1 % watery extract of them nearly corresponded to 0·1 and 0·4 % respectively referred to the dried substances.

When we look at Table X with this knowledge, we notice immediately that the content of active urease in soy-bean germs must be very high. It is higher than in the precipitated urease itself. In contrast to this fact the absence of activating power on dialysed urease interested me very much (see Table XI).

TABLE XI.

1·5 % urease dialysed in cc.	1 % germ-extract in cc.	1 % kernel-extract in cc.	1 % shell-extract in cc.	N/10 NH_3 developed in cc.
5	—	—	—	0·13 in 16·5 hours
5	2	—	—	3·05 ,,
5	—	2	—	0·83 ,,
5	—	—	2	0 ,,
—	2	—	—	3·29 ,,
—	—	2	—	0·67 ,,
—	—	—	2	−0·02 ,,
5	—	—	—	0·04 in 17·7 hours
5	2	—	—	2·65 ,,
—	2	—	—	2·63 ,,

The process of germination of soy-bean is to be compared with dialysis, because in both processes the exchange of substances takes place between the urease or the bean-kernels and the surrounding water through a vegetable membrane. As a result, the co-enzyme of the germs and kernels was lost through the germination.

7. *Upon which constituent of urease, the diffusible or non-diffusible one, does acid or alkali act?*

An investigation was made to see which factor is influenced by these reagents. N/1000 HCl was allowed to act on urease for ten minutes, and was then neutralised by NaOH. Co-enzyme (fresh urease) and urease without co-enzyme (urease heated for an hour to 80° C., see following experiments) were added to the above-mentioned mixture and the activity measured with the result shown in Table XII.

It is to be noticed that the data concerning the inhibitory effects of various substances were not always uniform. Therefore in such cases I will describe the results of parallel experiments as obtained.

From this table one can easily see that N/1000 HCl only destroyed the diffusible part, and the remaining urease was put in action again by the

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TABLE XII.

H_2O in cc.	0·5 % urease in cc.	N/200 HCl in cc.	Neutralised by addition of N/200 NaOH in cc.	H_2O in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 20 hours in cc.
45	5	—	—	12·5	—	5	22·10
35	5	10	10	2·5	—	5	0·10 } 0·07
35	5	10	10	2·5	—	5	0·05 }
35	5	10	10	1·5	1	5	9·53 } 9·75
35	5	10	10	1·5	1	5	9·98 }
35	5	10	10	0·5	2*	5	−0·02
40	—	10	10	0·5	2*	5	−0·03
40	—	10	10	1·5	1	5	0·20

* Heated to 80° C. for an hour.

addition of co-enzyme but not by the addition of the urease heated to 80° C. In a similar manner the effect of alkali was investigated. N/50 NaOH was allowed to act on urease for an hour.

TABLE XIII.

H_2O in cc.	0·5 % urease in cc.	N/10 NaOH in cc.	Neutralised by addition of N/10 H_2SO_4 in cc.	H_2O in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 development in 27·7 hours in cc.
45	5	—	—	11	—	5	22·52
35	5	10	10	1	—	5	7·17 } 6·85
35	5	10	10	1	—	5	6·53 }
35	5	10	10	—	1	5	17·35 } 17·17
35	5	10	10	—	1	5	17·00 }
40	—	10	10	—	1	5	0·74

From this table we may safely conclude that NaOH destroyed the greater part of the co-enzyme and this co-enzyme lost could be restored by the addition of fresh urease. I regret the data were not decisive, but those of Table XV supply this deficiency.

If acid and alkali act on co-enzyme only, we may assume that these substances will not be able to affect the dialysed urease, which is free from co-enzyme.

Experiments concerning this point are shown in Tables XIV and XV.

TABLE XIV.

H_2O in cc.	0·5 % urease dialysed in cc.	N/200 H_2SO_4 in cc.	Neutralised by addition of N/200 NaOH in cc.	H_2O in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 18·7 hours in cc.
45	5	—	—	11	—	5	0·16
35	5	10	10	1	—	5	0·00 } 0·00
35	5	10	10	1	—	5	0·01 }
35	5	10	10	—	1	5	2·87 } 2·89
35	5	10	10	—	1	5	2·91 }
40	—	10	10	—	1	5	0·29
45	5	—	—	10	1	5	3·00

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TABLE XV.

H_2O in cc.	0·5 % urease dialysed in cc.	N/10 NaOH in cc.	Neutralised by addition of N/10 HCl in cc.	H_2O in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 27·5 hours in cc.
35	5	10	10	1	—	5	0·03 } 0·02
35	5	10	10	1	—	5	0·01 }
35	5	10	10	—	1	5	3·41
40	—	10	10	—	1	5	0·71

From these tables we see that the previous hypothesis was confirmed. Dialysed urease itself was not inhibited by acid at all and could develop as much ammonia as the urease without the acid influence when fresh urease (co-enzyme) was added to them both. Acid and alkali therefore only destroy the co-enzyme but not the urease itself.

8. *What is the influence of heat on urease?*

Van Slyke and Cullen [1914] found that the optimum temperature for urease action was 55°; heating of urease to 60° for 30 minutes was ineffective, heating of urease to 70° for 30 minutes deprived it of 25 % of its activity, but heating to 80° for 30 minutes destroyed it completely. A first experiment was made with urease which was heated to 60° for half an hour.

TABLE XVI.

0·5 % urease heated to 60° for an hour in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 20·3 hours in cc.
5	—	22·31
5	1	22·28
—	1	0·44

TABLE XVII.

1·5 % urease dialysed for 5 days, in cc.	0·5 % fresh urea in cc. Not heated	0·5 % fresh urea in cc. Heated to 60° for half an hour	N/10 NH_3 developed in 17·7 hours in cc.
5	—	—	0·04
5	2	—	12·32
5	—	2	10·76
—	2	—	1·54
—	—	2	1·57

In Table XVI we note that the urease was not injured at all by heating to 60° for an hour. The heated urease solution contained obviously a sufficient amount of urease proper and co-enzyme to hydrolyse the amount of urea present. Accordingly, the addition of a small amount of urease proper and co-enzyme did not show any appreciable effect.

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But heating to 60° even for half an hour was not without effect on the urease system. As Table XVII clearly shows, some amount of the free co-enzyme was lost, and in regard to the capacity of reactivating dialysed urease the heated urease was inferior to the unheated.

Experiments were carried out with the urease which was heated to a higher temperature as follows:

TABLE XVIII.

0·5 % urease heated to 80° for an hour in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 20·3 hours in cc.
5	—	0·06
5	1	11·61
—	1	0·42

TABLE XIX.

0·5 % urease heated up to boiling point in cc.	0·5 % urease in cc.	N/10 NH_3 developed in 17·25 hours in cc.
5	—	−0·02
5	1	10·93
—	1	0·43

It is clearly shown in these tables that heating to 80° for an hour or heating just up to boiling point totally destroyed the co-enzyme in the urease solution both fixed and free. But the addition of co-enzyme (fresh urease) enormously revived the action of the urease.

By these experiments the statements of van Slyke and Cullen as to the effect of heat on urease were confirmed. Further the heating of the urease solution above 80° totally destroyed the co-enzyme but not the urease proper. But the free co-enzyme was somewhat labile, it was already somewhat injured by heating to 60° for half an hour.

As a natural consequence it occurred to me that dialysed urease should not be destroyed by heating since it has no co-enzyme. An experiment to test this was made:

TABLE XX.

0·5 % urease dialysed for 5 days, in cc.	0·5 % urease in cc.	N/10 NH_3 developed in 18·8 hours in cc.
5	—	0·26
5	1	4·57
—	1	0·06
Heated to 80° for an hour		
5	—	0·10
5	1	2·59
Heated up to boiling point		
5	—	0·02
5	1	1·68

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The conclusion was shown to be correct. Only the yield of ammonia produced by the dialysed urease was smaller if it had been heated. This difference may be ascribed to the total destruction of the co-enzyme which remained fixed. The heating up to boiling point, however, will very likely destroy a certain amount of urease proper.

9. *To what change is the diminution of the activity of urease during preservation to be ascribed?*

Since it has been shown that co-enzyme is in every respect very labile, it is probable that the diminution of the activity of urease during preservation is due to change of co-enzyme.

An experiment was made with urease which was kept with toluene in an incubator at 36°·5 for 15 days. The urease solution was 0·5 % and 5 cc. of it developed 22·6 cc. of N/10 NH_3 in 20·3 hours at the start.

TABLE XXI.

Old urease in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 18·7 hours in cc.
5	—	5·17
5	0·5	10·87
—	0·5	0·11

It is very likely that the co-enzyme was subjected to some change during the preservation, but the data are not sufficiently convincing. Whether the urease proper remained quite intact is also uncertain.

10. *Does blood serum contain co-enzyme beside "auxo-urease"?*

Margarete Falk [1914], and Jacoby and Umeda [1915] studied "auxo-urease" in blood sera which considerably accelerated the urease action. These statements interested me and suggested the attempt to find the co-enzyme of urease in blood.

TABLE XXII.

0·5 % urease dialysed in cc.	Serum of ox in cc.	0·5 % urease heated to 80° for an hour in cc.	0·5 % urease in cc.	N/10 NH_3 developed in 17 hours in cc.
—	—	—	2	1·70
—	1	—	—	−0·02
—	1	—	2	17·17
5	—	—	—	0·26
5	1	—	—	1·21
—	—	5	—	0
—	1	5	—	0·05
—	—	5	1	10·08

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As is shown in this table ox serum contains no urease, but a small quantity of the serum accelerates the urease action to an enormous amount; no true co-enzyme was however found in the serum. The urease which was heated at 80° for an hour lost its activity totally, but the addition of the serum could not revive the action at all, while the addition of a small quantity of the fresh urease (co-enzyme) restored the lost activity considerably. The dialysed urease employed was not quite free from the fixed co-enzyme, consequently the addition of the serum caused appreciable increase of activity. This effect was undoubtedly due to an accelerator in the serum, the "auxo-urease."

Discussion.

Since urease loses its activity by dialysis and recovers it by the addition of something contained in fresh solutions, we are justified in calling this something a "co-enzyme," in view of the present ignorance as to what a co-enzyme really is. The facts that it is destroyed by heat and by the action of acid, alkali, etc. suggest a protein nature, but its apparent passage through parchment paper negatives this suggestion, unless it be assumed that it requires for its activity the presence of some diffusible substance. But, again, the fact that addition of the dialysate is ineffective is remarkable and, on the hypothesis suggested, could only be accounted for by supposing that the protein or colloidal activator undergoes some irreversible change. What is distinctly proved is that what is called "urease" can be brought into an inactive state which can be restored to activity by the addition of a trace of some substance or substances which are contained in fresh extracts of soy-bean.

It is clear that further work is necessary to decide which constituents are to be called the enzyme proper and which the "co-enzyme." The possibility cannot be excluded that the residue in the dialyser might contain a substance capable of increasing the activity of fresh urease, although this does not seem probable.

Conclusions.

(1) The urease of soy-bean loses its activity on dialysis. The lost activity is restored by the addition of a small amount of fresh urease. This indicates that the fresh urease contains co-enzyme. The co-enzyme could not be separated, accordingly its nature is not yet known.

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(2) It is very likely that the co-enzyme is a system consisting of two groups of components, one of which is dialysable and the other undialysable. The dialysable component undergoes some irreversible change during dialysis.

(3) The co-enzyme consists of two parts, fixed and free.

(4) Heating and dialysis destroy the free co-enzyme first, then the fixed co-enzyme. The last portion of the fixed co-enzyme is found in the precipitate produced by dialysis, resisting the influence of heating and dialysis tenaciously.

(5) The inhibitory effects of heat, acid and alkali are exerted upon the co-enzyme, but not upon the urease proper.

(6) In germination, urease accumulates in the germs of the soy-beans in large proportion, but free co-enzyme is absent.

(7) Although ox serum has an accelerating power, it contains no substance which can be compared with the co-enzyme.

In conclusion I have pleasure in expressing my cordial thanks to Prof. Bayliss for his advice.

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LII. ON THE PREPARATION OF COLLODION MEMBRANES OF DIFFERENTIAL PERMEABILITY.

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In the course of a study of the active principle of the extract of a parasitic fungus, the writer [Brown, 1915] attempted the fractional dialysis of the extract. For this purpose he first employed membranes prepared by impregnating filter papers with gelatin according to the method of Bechhold [1907]. By varying the strength of the gelatin employed a series of membranes of different permeability was obtained, none of which however was able to hold back completely the active principle of the fungus. Recourse was had, therefore, to collodion membranes. As this technique was new at the time to the writer, extremely discordant results were at first obtained. It soon appeared that with the same collodion solution very great differences in the properties of the resulting membranes could be produced by variations in the mode of treatment. A revision of this technique was undertaken, involving on the one hand a critical examination of the methods hitherto described and on the other the elaboration of methods which, to the present knowledge of the writer, constitute a new departure in the preparation of collodion dialysers.

It is not proposed to enter fully into the somewhat scattered literature relative to collodion membranes. A fairly complete account of the older literature on the subject is given in a paper by Bigelow and Gemberling [1907].

In practice collodion membranes are employed in the form either of flat sheets or of thimbles. Each type has certain special advantages. Thus the former lends itself more readily to osmometric work inasmuch as it can

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easily be backed by some arrangement to take up the pressures developed; for purposes of dialysis the thimble form is simpler in manipulation as it does not require to be tied to any glass structure.

Collodion thimbles may be formed in two ways. A test tube is dipped into a collodion solution, and the resulting film left to undergo a certain amount of evaporation of the solvent. The film is then hardened by immersion in water, and the membrane so produced is stripped off by eversion. This is the method used by Malfitano [1904], by Duclaux [1907], etc. By other experimenters the membranes are formed inside the tube. The latter is filled with collodion solution which is then poured out, and the film of collodion remaining is allowed to drain for some time. The film is hardened by addition of water, and the resulting membrane readily shrinks away from the glass tube. This is the method described by Smith [1905], Bigelow and Gemberling [1907], Lillie [1907], Walpole [1915], etc.

Sheet collodion is prepared by pouring thin collodion solution over a glass surface which has been carefully levelled [Walpole, 1915] or on a mercury surface [Bigelow and Gemberling, 1907]. It is hardened in the usual way.

Numerous attempts have been made to prepare collodion membranes of uniformly varied permeability. These now call for detailed mention.

Bechhold [1907] prepared collodion films by impregnating filter papers with solutions of different strengths of collodion in glacial acetic acid. Impregnation was carried out *in vacuo*, and the filter papers after draining for a short time were hardened by immersion in water. The permeability of the membranes obtained was found to vary inversely with the strength of the collodion solution used.

Bigelow and Gemberling [1907] used a solution consisting of 3 g. commercial pyroxylin in 75 cc. ether + 25 cc. alcohol. They varied the permeability by varying the time allowed for evaporation of the solvents. No means of standardising this process were described.

Malfitano [1910] observed that the permeability of collodion thimbles diminished as they were allowed to dry in air; also that it could be diminished by heating the membrane in water. With similar treatment in other respects, a collodion richer in alcohol gave a more permeable membrane than one rich in ether. No details of a systematic nature were given.

Schoep [1911] describes a method which does not seem to have received the recognition that it deserves. He prepared membranes from pyroxylin-

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castor oil solutions in alcohol-ether. The following represents the type of formula used by him:

Pyroxylin	4 g.
Castor oil	3·5 g.
Alcohol and ether	92·5 cc. (alcohol and ether in ratio 1 : 8).

In order to vary the permeability of his membranes, Schoep added glycerol in various quantities to the pyroxylin-castor oil solution. The membranes were then formed inside a glass tube and allowed to drain and dry to constancy. On testing these membranes he found that their permeability increased with increasing glycerol concentration used.

Walpole [1915] relied mainly upon sheet collodion prepared by pouring a 3·5 times diluted stock solution of Schering's celloidin [13·45 per cent. by weight in equal parts of absolute alcohol and ether—thus approximately a 4 % solution] over a horizontal glass plate. The amount of solution used and the area of the resultant membrane being known, the amount of nitro-cotton per sq. cm. could be determined. This quantity (m) is used in describing the membrane. In order to obtain membranes of varied permeability Walpole varied the drying period. To determine how far drying had proceeded, he took sample portions of the film, washed them in water, and determined their thickness by means of a micrometer. With the same film, the thickness of the resulting membrane decreased as evaporation proceeded, so that by determining the former the progress of evaporation could be followed. Walpole found that the membranes so obtained could be described fully, for purposes of reference and reproduction and as a guide to their properties, by stating the kind of nitro-cotton used, the weight of it per sq. cm. they contained, and the "wetness," i.e. the ratio of the weight of a piece of film to its weight when dried to constancy.

The method of varying the permeability discovered by the writer is as follows. Collodion membranes are prepared and then completely air-dried whereby they become highly impermeable; they are then placed in solutions of alcohol in water for a suitable time. When subsequently washed in water these membranes are found to show a permeability which decreases regularly with the strength of the alcohol employed.

Thus while in the methods of manufacture hitherto employed, successful grading was a matter of skill which was not always definable, the present method is merely mechanical and the only skill required is in the making of the initial uniform air-dried membranes.

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Standard method of preparation of air-dried membranes.

An 8 % solution of Schering's celloidin in an equal mixture (by volume) of absolute ethyl alcohol and ether is used. The membranes are formed on the outside of a test tube (1 cm. diam.). The latter is dipped in the collodion solution to a depth of 8–10 cm., and after a few seconds' draining is placed in a vertical position inside an inverted conical flask ($\frac{1}{2}$ litre capacity), being held by an ordinary cork which fits the neck of the flask. The purpose of the flask was in the first instance to limit the evaporation of the solvents so as to ensure an even film of collodion. By manipulation of the tube after insertion into the flask the length of the membrane can be increased. Drainage is allowed to proceed for 5 minutes after which the tube is withdrawn and *immediately* immersed in water. After a time (about 1 minute) the membrane is stripped off by eversion. For this purpose use is made of the "runnels" of collodion, which have by now become solidified. These on being cut across serve as handles in pulling the membrane off. The membranes are then trimmed, washed for a short time in water, and laid out to dry at laboratory temperature. Drying takes place with shrinkage and is completed overnight. The membranes are now ready for the alcohol treatment.

The shrinkage consequent on drying is a factor of very considerable importance in the present technique. A membrane which dries with excessive shrinkage will show at the finish a diminished surface over which diffusion may take place, along with increased thickness, and will not afford results comparable with those of a less shrunken membrane. Great care has therefore been exercised in examining the factors determining this shrinkage and in devising means for its proper control. What these factors are will appear from the following.

Characters of a normal membrane previous to drying off.

The characters which were examined in this connection were:

(1) permeability;

(2) water-content;

(3) thickness, before and after drying; shrinkage on drying, both longitudinal and circumferential.

A normal membrane is permeable to all crystalloids and allows slow diffusion of dextrin. It is more permeable at the closed end and may be relatively impermeable towards the open end. This is shown by the fact

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that with certain dyes the inner surface of the membrane stains more rapidly in the neighbourhood of the closed end than towards the opening. Again the permeability of the outer surface is greater than that of the inner surface.

After washing in water, these membranes contain from 39·5 to 43·0 % of water (in terms of total weight).

It is obvious from the foregoing that with membranes of the present type the wetness gives no certain indication of the permeability as the membrane is not uniform on both sides.

The following method was adopted in measuring the shrinkage relationships of these membranes. The membrane was split from top to bottom along one side; the circumference was determined at top (open) and bottom (closed) ends, and also the thickness (by means of a micrometer) across the top, middle and bottom. In order to determine the degree of longitudinal shrinkage, a distance of 4 cm. was measured and marked by cuts in the long direction of the membrane. A repetition of these measurements in the case of the air-dried membrane gave data by which the shrinkage relationships could be followed.

The following series of measurements is typical of the present type of membrane (viz. a membrane which was drained 5 minutes in the flask and was immediately immersed in water). In the micrometer readings, each unit is equivalent to 5 μ.

Membrane before drying.

Circumference = 4·53 cm. at top; 4·50 at base.

Micrometer readings.

5·2, 5·8, 5·4, 5·5, 5·3, 5·0, 5·1 near top.	Av. = 5·33.
5·7, 5·7, 5·6, 5·6, 5·8, 6·0, 5·9 near middle	Av. = 5·76.
6·3, 6·3, 6·6, 6·3, 6·2, 6·4, 6·2 near base.	Av. = 6·33.

After drying.

Circumference = 4·15 cm. at top; 4·05 at base.
Marks 4 cm. apart have shrunk to distance 3·63 cm.

Micrometer readings.

4·0, 4·0, 4·0, 4·0, 4·0, 3·9, 3·7 across top.	Av. = 3·95.
3·6, 3·7, 3·5, 3·6, 3·5, 3·5, 3·4 across middle.	Av. = 3·55.
3·3, 3·3, 3·2, 3·2, 3·2, 3·2, 3·2 across base.	Av. = 3·23.

In connection with this series of figures, the following points are noteworthy:

(1) At the same level of the thimble there is a variation in thickness, both in wet and in air-dried membranes. This variation is greatest near the open end. This can be ascribed to unevenness of drainage. It is better to discard the top part of each membrane.

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(2) There is a variation in thickness from base to top of the same membrane.

In the case of air-dried membranes, thickness is found to increase from closed to open end. From a large number of observations, this rule has been found to be general. No such rule is however applicable to the corresponding undried membranes. Thus in the preceding case, the undried membrane was thicker towards the closed end, but nevertheless the closed end became the thinnest part of the membrane when the latter was dried off. Correlated with this we find the basal region to be the most permeable part of the wet membrane. In many cases however the undried membranes are found to show the same increase in thickness from the closed end upwards. The cause of these variations will be discussed below.

(3) The shrinkage relationships may be summarised thus:

Length before drying	= 100	Length after drying	= 90·75
Circumference before drying	at top = 100 at base = 100	Circumference after drying	at top = 91·6 at base = 90·0
Thickness before drying	at top = 100 at base = 100	Thickness after drying	at top = 74·1 at base = 51

Measurements of the above type have been carried out for a large number of membranes. The following general rules can be drawn:

(*a*) Longitudinal and circumferential shrinkage are approximately the same. Shrinkage is slightly greater at the closed end. Shrinkage in different membranes of the same type is remarkably uniform; thus of more than 30 measurements made, all lay within the limits 87 and 92, the majority lying between 88 and 90.

(*b*) Shrinkage in thickness is more variable. The thickness of the dried membrane varies from 50 to 75 % of the undried. Shrinkage in thickness is always greater than superficial shrinkage. This anisotropy in respect of shrinkage is invariable with these membranes.

The results so far obtained may be conveniently summarised at this stage.

By following the method described on p. 594, membranes can be obtained which on being air-dried present the following features: thickness = $20 \pm 5\,\mu$; tendency to greater thickness towards the open end; longitudinal and circumferential shrinkage very constantly 10 %.

Before drying, these membranes show a different permeability in their different parts; in this state therefore they are of no use whatever in cases where anything like exactness is required in dialysis work.

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To attain a high degree of uniformity of the air-dried membranes certain precautions must be taken. The most important of these will appear from the following discussion of the effect of varying the conditions on the nature of the resulting membrane.

(i) *Effect of allowing free evaporation of the solvents.*

In the method of preparation described above, the membranes after draining in the flask are immediately plunged into water. The effect produced by even a short sojourn in the free atmosphere of the laboratory is very remarkable. This will appear from the following data:

The membrane is drained for 5 minutes in flask; on taking out it is held for 1 minute in air. Such a membrane will be described by the notation (5, 1); by the notation (5, 0) is meant a membrane which after 5 minutes' draining in the flask is immediately plunged into water.

Measurements of a (5, 1) *membrane.*

Circumference at top and bottom = 4·55 cm.
Micrometer readings 17·0, 17·0, 19·0, 18·7 across top. Av. = 17·9.
17·5, 16·3, 17·5, 18·9 across middle. Av. = 17·55.
15·3, 15·0, 15·9, 15·1 across base. Av. = 15·3.

After drying.

Circumference = 3·2 cm. at top; 3·1 at base.
Distance between 4 cm. marks = 2·8 cm.
Micrometer readings 8·0, 7·0, 6·5, 6·5 across top. Av. = 7·0.
7·0, 7·0, 6·3, 6·8 across middle. Av. = 6·8.
5·7, 6·1, 6·1, 6·1 across bottom. Av. = 6·0.

Thus the shrinkage relationships are summarised in the following table.

Length before drying		= 100	Length after drying		= 70·0
Circumference before drying	at top	= 100	Circumference after drying	at top	= 70·3
	at bot.	= 100		at bot.	= 68·1
Thickness before drying	at top	= 100	Thickness after drying	at top	= 39
	at bot.	= 100		at bot.	= 39

There is thus in this case excessive shrinkage on drying. Membranes of this type are before drying highly permeable—much more so than membranes of the type (5, 0). They are slowly permeable to aniline blue, strongly permeable to dextrin and allow crystalloids to pass with great rapidity. Correlated with this they show a high water-content; thus weight of dry membrane = 100; weight of wet membrane = 170.

The above figures are typical of all membranes of this type. The longitudinal and circumferential shrinkage borders very constantly on 30 %; the shrinkage in thickness approximates closely to 60 %.

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A comparison of the measurements of a (5, 0) and a (5, 1) membrane is instructive. The unit of thickness is, as before, 5 μ.

(5, 0). Before drying. Thickness = 5–7.
After ,, ,, = 3–4.

Longitudinal and circumferential shrinkage = 10 %.
Shrinkage in thickness = 25–50 %.

(5, 1). Before drying. Thickness = 15–18.
After ,, ,, = 6–7.

Longitudinal and circumferential shrinkage = 30 %.
Shrinkage in thickness = 60 %.

It will be noticed that while shrinkage in thickness is greater in membranes of the second type, the final thickness of the latter when dried is about twice that of the former.

It will thus be seen that unequal evaporation of the solvents produces unequal shrinkage in the resultant membranes. The fuller discussion of these results will be postponed until the variation of a second factor has been examined.

(ii) *Effect of varying the time of drainage.*

The time of drainage within the flask was varied from 1 minute up to 10 minutes, and the resulting membranes tested as to their permeability, thickness in the wet condition, and shrinkage on drying. The following table gives the figures obtained. The notation in the case of the membranes is that already described (p. 597).

Membrane	Thickness when wet	Longitudinal and circumferential shrinkage	Thickness when dry
(1 0)	6–8	5–6 %	4·4–6·3
(1 ½)	17–19	20 %	5·7–6·9
(1 1)	19–20·4	25–27·5 %	6·7–8·0
(3 0)	6·3–7·9	7·5–10 %	3·7–4·8
(3 1)	17·0–20·8	28–30 %	6·2–7·2
(5 0)	5·3–7·1	8–12 %	3·3–4·5
(5 ¼)	14	22·5 %	5·1–5·7
(5 ½)	13·5–18	20–25 %	5·4–7·8
(5 1)	16·7–20·4	30–32 %	6·0–7·4
(5 2)	20·8	30 %	7·8
(10 0)	5·0–5·25	11–13 %	3·0–3·1
(10 1)	12·6–13·6	30 %	5·1–5·4

From the above table it is clear that by prolonging the period of drainage a slow increase of the shrinkage obtained on drying the resultant membrane takes place; that when once the membrane is taken out and allowed to

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evaporate freely, a rapid change sets in, and the shrinkage soon reaches as much as 30 %. In this technique therefore it is imperative that the membranes be taken from the flask and immediately immersed in water. The rapid execution of this process involves a certain amount of dexterity only attainable by practice. The writer has convinced himself that it is quite possible to reduce the shrinkage variation to less than 5 %. Should it be considered necessary to bring this factor under better control, it is obvious that an arrangement could be set up whereby the films could be hardened without being exposed at any time to rapid evaporation of the solvents in the open air.

By lengthening the time of drainage the thickness of the resultant membrane diminishes, but only slowly. Even after 10 minutes' draining, the membranes when dried are thicker at the open end than near the closed end. Uniformity in thickness is a condition which is approximated to only after very prolonged drainage.

The membranes which have been allowed to evaporate freely for some time all show high permeability. In the case of membranes which are immediately transferred to water, permeability increases with time of drainage. Thus a (1, 0) membrane is impermeable, except at the closed end, to neutral red; a (10, 0) membrane shows a fairly uniform medium permeability throughout. With intermediate drainage, the membranes show a gradation from a more permeable region near the closed end to a less permeable region near the open end.

A membrane of the (1, 0) type when placed in water becomes strongly milky in appearance except just at the closed end which is clear. By longer drainage, the milkiness is reduced, and in the case of a (5, 0) membrane becomes very slight. Membranes of the strongly shrinking type are always glass-clear.

It is convenient at this point to explain the principles underlying the phenomena above recorded. For this purpose it is necessary to anticipate somewhat.

Membranes prepared from a solution of collodion in ether alone are highly impermeable, are white, have a low water-content, and show a very small shrinkage on drying. This statement applies all the more markedly when practically no ether is allowed to evaporate from the film before it is placed in water. Membranes made from a solution of collodion in alcohol alone are, on the other hand (unless a large proportion of the alcohol be allowed to evaporate), highly permeable, are clear, have a high water-content

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and undergo strong shrinkage on drying. The various types of membrane obtained above represent intermediate stages of this series.

Now when a film of collodion on a test tube is exposed to free evaporation, the first result is the rapid diminution of the ether content. The membrane therefore lies near the alcohol end of the series, and a highly permeable, strongly shrinking membrane is obtained. Membranes of the type (1, 1), (3, 1), (5, $\frac{1}{2}$), (5, 1), (10, 1) are nearer to the alcohol end of the series, those of type (1, 0), (3, 0), (5, 0), (10, 0) are nearer to the ether end. That the (1, 0) membrane approximates more closely to an ether-collodion membrane than does the (10, 0) is due to the fact that the atmosphere inside the flask requires some time to reach equilibrium, and also to the fact that with the apparatus used a slow leakage of ether took place at the neck of the flask. That the basal (closed) end approximates more closely to the alcohol type of membrane than does the other is to be set down to the fact that in the earlier stages of drainage when evaporation is most rapid the closed end of the film (which is uppermost) has reached its limiting thinness, whereas the lower portion is protected by the excess collodion which is flowing past. This inequality, as has been already stated, is greatly diminished after about 5 minutes' drainage.

It will conduce to clearness at this stage if the essential features of the technique described above be emphasised. This is most conveniently done by stating the reasons which lead the writer to recommend each particular detail of the method described on p. 594.

It should be stated first of all that membranes of the strongly shrinking type are to be avoided for reasons of efficiency in dialysis. With equal dimensions at the start, the capacity of a membrane which has shrunk by 30 % bears a ratio $7^3 : 9^3 = 1 : 2{\cdot}1$ to that of one which has shrunk by 10 %—i.e. it is less than half. The thickness of the much shrunk membrane is also greater, so that with the same intrinsic permeability, diffusion is slowed down. It need hardly be added that in setting up any series of graded dialysers, membranes showing different degrees of shrinkage must be avoided.

Use of the conical flask. When a test tube is dipped into an 8 % collodion solution and withdrawn, a drop of collodion forms at the closed end of the tube. When the tube is placed vertically with closed end upwards, this drop must be made to travel the whole length of the film, otherwise extreme irregularity in thickness of the membranes results. When the films are allowed to evaporate freely there is a tendency for the excess collodion to stick half way, and very uneven drainage results. This tendency is

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especially marked in warm weather. Again, even if drainage is fairly uniform, the membranes are found on drying to be of the strongly shrinking type. The use of the flask is to obtain even drainage along with a minimum of shrinkage on drying.

Strength of collodion used. With increase in concentration above 8 %, a tendency to uneven drainage sets in. Thus 10 % collodion after 5 minutes' drainage gives distinctly less uniform membranes than does an 8 % solution. With a 5 % solution, even drainage is soon secured. The membranes are however more liable to tear on stripping from the tube, and are found to shrink strongly. The latter feature is due to the fact that on account of the comparative fluidity of a 5 % solution, only a small amount of solution is carried into the flask on the tube, and consequently a considerable percentage of the solvent (especially ether) is evaporated before the atmosphere of the flask becomes saturated.

Time allowed for drainage. For the membranes normally employed, a period of 5 minutes' drainage was allowed.

For the first 1–2 minutes, drainage is still proceeding rapidly. From 3 minutes onwards only a very slow drainage takes place. Membranes which have drained 3 minutes show greater variation between closed and open ends than do membranes of the normal type. They also show a much greater tendency to milkiness. The latter objectionable feature is reduced to very small dimensions in the case of membranes which have drained 5 minutes. The only objection to drainage longer than 5 minutes is the time thereby taken up.

In carrying out the routine of preparing membranes, the experimenter cannot afford to abate any consideration which tends to uniformity. Each process in the manipulation should be standardised. In particular, the membranes should be made at a standard temperature (not higher than 20° C.), and as nearly as possible the same amount of solution should be taken up by the test tube in each case.

Summary. By carrying out the above routine, membranes can be obtained which show a shrinkage on drying of 10 ± 2 %. The dried membranes vary in thickness from 15 μ at the closed end to 20–25 μ at the open end; at any given level in the same membrane a variation in thickness of 10 % is liable to occur.

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Treatment with alcohol.

The stock alcohol (ethyl) employed was of specific gravity 0·7956 (at 16° C.), corresponding thus to 99·36 % absolute (by weight).

By an x % alcohol in the following account is meant an alcohol-water mixture containing x cc. of stock spirit + $(100 - x)$ cc. water.

The air-dried membranes are soaked in the alcohol mixtures for 24 hours at 20° C.; they are then transferred to distilled water and thoroughly washed over a period of 1 day. They are then ready for use.

When uniform air-dried membranes are used, the permeability is determined by the concentration of alcohol employed. It may thus be fully specified in terms of the alcohol percentage. By the phrase "a 75 % membrane" is meant an air-dried membrane which has been soaked in 75 % alcohol for 24 hours at 20° C., and subsequently washed in water.

The series of membranes treated with increasing percentages of alcohol is thus a series showing gradually increasing permeability. The limits of the series are on the one hand 0 %, on the other about 97 %, above which point the membrane goes into solution. The most permeable membrane that has been systematically used is a 96 % one; 97 % membranes have also been prepared but are extremely fragile and apt to rupture. Membranes more permeable still have been prepared by allowing a shorter imbibition period in 98 % alcohol. It is possible therefore to extend the series, thereby obtaining extremely permeable membranes, but they are of doubtful value unless means be taken to support them and protect them from mechanical injury.

In the following work these membranes were set up in specimen tubes of 1·7–2 cm. diameter. In cases where estimations of diffusion were made, tubes of 1·8 cm. diameter were alone used. The membranes were filled with the solution to be tested to a distance of about 1 cm. from the top, and the space between the membrane and the tube filled to the same level with water.

The following substances were used:

$NaCl$(m), NH_4Cl(m), Na_2SO_4(m), $CuSO_4$ (sat.), $K_4FeC_6N_6$ (sat.), $K_2C_2O_4$(m), glucose (m and m/5), picric acid (sat.), $KMnO_4$ (0·5 %).

Bismarck brown, methylene blue, eosin, neutral red, safranine, aniline blue, Congo red, night blue (all 0·1 %).

Dextrin (0·5 %), starch (0·1 %), litmus (Kahlbaum).

All the solutions were made up in water.

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In order to prove that a continuous gradation in permeability is obtained by the alcohol treatment, four main methods were employed.

I. Using a suitable crystalloid and membranes within a given range, the amount of endosmosis of water in a given time increases with the strength of alcohol used.

This method is capable of great elaboration, viz. by constituting the membrane an osmotic cell with capillary tube attached, and measuring the rate of endosmosis of water. This method of testing the variation of permeability with alcohol treatment, and the degree of uniformity of membranes treated in the same way, has not been carried out in detail as yet and only a few rough measurements of the alteration of level between the liquid inside the membrane and the water outside are at present available. In most cases no measurement was taken, but a note made to the effect that rate of endosmosis increased with strength of alcohol used. These measurements were made incidentally to determining the permeability of these membranes to various salts.

The following figures illustrate the type of result observed:

0 %, 60 %, and 70 % membranes set up with molar potassium oxalate.

After 24 hours, endosmosis of water in	0 %	= 0·2 cm.
,, ,,	60 %	= 3·5 ,,
,, ,,	70 %	= 4·6 ,,

The increased endosmosis in 70 % takes place in spite of a slight passage of the oxalate through the membrane. In fact, increased endosmosis of water with strength of alcohol used takes place long after the membrane has become quite permeable to the osmotic substance. In such a case, of course, the endosmotic effect will prove to be transitory.

II. Within certain limits, as regards nature of membrane and nature of dye, the amount of staining of the membrane in a given time increases with the strength of alcohol used.

This method has proved of great value, especially for revealing what amount of irregularity in permeability may exist in different parts of the same membrane. The present test is certainly the most accurate from this point of view that is known to the writer. Though much use has been made of this test, only the cruder outlines are as yet worked out. The method lends itself readily to exact colorimetric work.

The following account of a particular experiment will illustrate how the method works out:

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50 %, 60 %, 70 %, 80 % membranes set up with neutral red solution inside, water outside.

After 1 hour's staining, all membranes are thoroughly washed in water.

A continuous gradation in staining effect is seen, the latter being least in the 50 % and greatest in the 80 % membrane. The membranes are again set up with neutral red and left for 24 hours. After this time, the dye has passed through the 80 % membranes. On cutting all the membranes open, the difference in staining between the 70 and 80 % membranes is now found to be slight; a marked difference is still shown between 50 and 70 %. Even after prolonged staining, this difference is not effaced—that is, e.g. the intensity of staining of a 60 % membrane never reaches that of a 70 % one.

From an extended series of experiments of this sort, a continuous gradation in general staining effect with varying strength of alcohol has been established. This is readily done in the case of 0 %, 30 %, 40 %, 50 %, 60 % and 70 % membranes with neutral red solution; by 24 hours' staining with aniline blue, a similar gradation can be established in the case of 70, 75, 80, 85 and 90 % membranes. Staining of 80, 85, and 90 % membranes with neutral red takes place too rapidly to allow of a satisfactory differentiation.

In applying this method to the detection of irregularities in permeability of different parts of the same membrane, the latter should be stained with a dye which is unable to pass through it. Thus neutral red was employed in the case of 30–70 % membranes; aniline blue for 70–90 % membranes. In cases where the dye is able to pass through the membrane, the staining rapidly becomes so intense that variations are no longer noticeable.

The following are the results obtained:

(1) With 70 % and higher membranes, staining is completely uniform, apart from a limited number of small spots at which the membrane is less permeable.

(2) With membranes less than 70 % there is, in addition to the above irregularity, a tendency to uneven staining, the membrane being more permeable near the closed than near the open end.

(3) When the outside of the membrane is tested in this way, greater irregularity may occur. This irregularity is correlated with the presence of milkiness, the milky patches staining more strongly. This effect is shown equally well in the air-dried (0 %) membranes, and with such a dye as aniline blue. On cutting such membranes and examining microscopically, the staining effect is seen to stop in the middle of the membrane.

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With glass-clear membranes the degree of staining of the outer surface is uniform and equal to that of the inner surface.

It may be remarked here that the presence of a number of small less permeable spots has been observed in the case of membranes of all kinds, no matter how made and no matter what the duration of the alcohol treatment. Their cause is unknown. From their small size, their effect on the general permeability of the membrane is negligible.

III. The amount of a particular substance passing through in a given time increases with strength of alcohol used.

Increased permeability with varying strength of alcohol has been followed over a range of 30 % to 96 % by measuring the rate of diffusion through the various membranes of certain substances. The membranes were set up as usual, the containing specimen tubes being chosen of equal diameter. The diffusing substance was placed inside, and the external space filled to the same level with water. The substances employed were such that no appreciable disturbance of levels (and volumes) was produced by endosmosis of water. Diffusion was carried on in a thermostat at 20° C.

(*a*) Diffusion of potassium permanganate (0·5 % solution). The amount diffused through in 24 hours was determined by titration with a 0·1 % $FeSO_4$ solution in dilute H_2SO_4.

Membranes—a double series of 30, 40, 50, 60, 70 %.

In all cases the tubes were filled to the same height, and at the end of the diffusion period the amount of solution inside the tube and outside the membrane approximated closely to 5 cc.

		Membrane				
		30 %	40 %	50 %	60 %	70 %
cc. of 0·1 % $FeSO_4$	Series *A*	0	1·6	10·6	31·2	76·5
	Series *B*	trace (< 0·10)	1·55	8·8	32·5	70·2

The difference between 60 % and 70 % is much greater than the figures represent. At the time of examining, equilibrium had nearly been reached between the outside and inside solutions.

The grading can also be seen by noticing how long a time elapses from filling the membranes till the first traces of colour appear in the liquid outside.

Thus in the case of the foregoing membranes, this happened within 1 minute in both cases with 70 %; in 4–5 min. in both with 60 %; in 25 min. in both with 50 %; in about 2 hours in both with 40 %; while in the case of 30 % membranes, nothing to a trace appears in 24 hours.

On washing these membranes staining is seen to have taken place; on

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again air-drying them they are found to be impermeable to permanganate. The presence of holes is thereby excluded. It is probable that the membranes are slowly attacked—at least the permanganate becomes turbid when kept in a membrane for about a week.

(*b*) Over the range 70 to 95 %, the grading can be followed by using methylene blue. Over the range 70–80 % this has been done in considerable detail (see Uniformity tests below). Over the wider range grading is shown by the time required for the dye to appear in the outside liquid. The following figures illustrate this point:

70 %	75 %	80 %	85 %	90 %	95 %
about 6 days	1 day	8–12 hours	4 hours	70 min.	12 min.

(*c*) Over the range 85 to 94 %, with variations of 1 % in alcohol treatment a continuous gradation of permeability can be shown by using dextrin as diffusing substance.

A dextrin solution of 0·5 % was used and dextrin was tested for in the external solution by adding 1 drop of N/10 iodine. After 24 hours, no trace appears in 85 %; a trace in 87 %, and this becomes stronger in each successive member of the series. The gradation becomes more rapid towards the upper end of the series.

(*d*) Over the range 92–96 %, the variation of permeability may be shown by use of neutral litmus.

After 10 days' dialysis, the following table gives the strength of dye in the external solutions:

92 %	93 %	94 %	95 %	96 %
0	trace	0·08	0·13	0·33

the internal solutions being of strength 1.

IV. The space relationships of the membrane (amount of water, swelling in all three dimensions) vary continuously with strength of alcohol used.

Gradation is shown over the whole range by measurement of the *water-content*. In making these determinations, the closed end of the membranes was cut away, and the cylindrical portion left was cut open. In determining the wet weights, both surfaces of the membrane were dried rapidly between filter paper; weighing was carried out in a closed bottle.

The gradation is shown in the following table. In each case the weight is given in terms of an original air-dry weight of 100. (Air-dried membranes contain about 3 % water.)

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Membrane %	Wet weight after washing	Weight on again drying
0	106·0	100·0
50	111·0	99·3
70	115·6	98·6
80	122·4	98·2
90	141·9	97·0
92	160·5	93·8
94	208·5	89·1
96	387·8	74·8

A parallel series of measurements of the strongly shrinking (5, 1) membranes gave essentially similar results, except that the water percentage in the case of these tended to be higher near the upper end of the series than that of the preceding type.

It will be noticed that the increase in water-content is gradual at first and becomes extremely rapid above 94 %; also that the membrane loses in dry weight with the treatment; that this loss is slight up to 90 %, but becomes very considerable at 96 %.

In the same way the length, circumference and thickness of the air-dried membranes increase continuously when the latter are immersed in alcohol solutions of increasing strength. The following tables show these effects.

(α) *Membranes which show* 10 % *shrinkage on drying.*

The measurements of L (length) and T (thickness) are given on the basis of a length and thickness in the air-dried condition of 100. Longitudinal and circumferential extension have never been found to differ appreciably.

Membrane %	In alcohol before washing		After washing		After drying	
	L	T[1]	L	T	L	T
0	101·7	—	101·7	100·0	100·0	100·0
50	103·7	—	102·0	108·3	97·8	102·8
70	105·8	—	102·0	110·5	—	105·2
80	108·6	—	103·3	118·9	96·7	105·4
90	112·9	—	107·0	162·2	94·7	108·1
92	115·8	—	107·7	200·0	92·1	114·3
94	119·7	—	111·7	280·0	89·5	116·7
96	132·1	—	120·9	362·0	76·7	*

[1] Readings not taken on account of softness of membranes, especially in the higher percentages of alcohol.

* Becomes very crinkled; micrometer reading valueless.

The features brought out by Tables (α) and (β) apart from the continuous gradation are:

(1) Swelling is anisotropic. Thus in 94 % alcohol, the longitudinal extension is 12 %; the extension in thickness is 180 %. This applies to both sets of membranes.

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(β) *Membranes which show* 30 % *shrinkage on drying.*

Membrane	In alcohol before washing		After washing		After drying	
%	*L*	*T*[1]	*L*	*T*	*L*	*T*
0	102·0	—	101·0	103·5	100·0	100·0
50	104·4	—	[100·5]	107·0	[96·8]	*
70	107·4	—	102·3	124·5	96·7	*
80	108·3	—	102·5	129·6	95·5	*
90	119·7	—	110·0	150·0	94·1	111·0
92	126·8	—	118·3	186·0	—	112·0
94	138·4	—	125·6	300·0	87·2	117·5
96	157·3	—	139·1	410·0	65·1	*

[1] Readings not taken on account of softness of membranes, especially in the higher percentages of alcohol.

* Impossible to obtain a reliable measurement on account of crinkling on drying.

(2) A normal (10 % shrinking) membrane is restored to its original length and circumference by immersion in alcohol of about 94 %. The thickness and, from table on p. 607, the water-content are much increased. Correlated with this is increased permeability of a 94 % membrane as compared with that of the membrane as originally prepared.

(3) With membranes of both types, longitudinal extension is negligible in membranes of 80 % and under; in these the effect of the alcohol treatment shows itself mainly in increase in thickness.

(4) With the higher percentages of alcohol, the strongly shrinking membranes show a higher extension than do the corresponding members of the normal series. Nevertheless the original dimensions are only reached at a higher alcohol percentage, viz. 94–96 %.

(5) The higher the percentage of alcohol employed, the greater is the subsequent shrinkage in length on drying (as shown by comparison of 2nd and 3rd *L* columns in both tables). This increased longitudinal shrinkage is compensated in part by increase in thickness on drying.

While the size relationships above described afford a very complete proof of the continuity in the series of membranes produced by alcohol treatment, their importance must not be over-emphasised. Collodion is a plastic substance, and therefore expansion takes place in the direction which is easiest. The anisotropy above observed is to be explained in this way. It does not at all mean that any kind of structure is to be postulated with regard to the membrane. The higher coefficient of longitudinal expansion of thick than of thin membranes is also to be explained in the same way—the thicker the membrane the more does it tend to expand isotropically, and similarly the thinner the membrane the greater is the tendency for expansion to be

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anisotropic. As an example of the latter may be cited some experiments with very thin membranes prepared in a way to be described later in which a longitudinal extension of 3–4 % was accompanied by an increase in thickness of 200 %.

In accordance with this principle, the permeable membranes obtained after alcohol treatment are more uniform in thickness than the dried membranes from which they were derived, and the tendency to uniformity is greater the higher the alcohol percentage.

Uniformity tests.

The diffusion experiments with potassium permanganate tabulated on p. 605 indicate the type of agreement obtaining between the corresponding members of two similarly treated series. More detailed tests of the same type were carried out within the range 70–80 % with methylene blue.

In one experiment, 15 membranes prepared in succession on the same day (membranes not selected in any way and of ordinary uniformity) were treated, 3 in 70 % alcohol, 3 in 72·5 %, and so on with 2·5 % intervals to 80 %. These were set up with methylene blue solution, and the rate of diffusion estimated from day to day by comparing the external liquids with a dilution series of the original methylene blue solution. The members of this series were of strength—1, 2, 4, 8, 16, 32, 64, 128, 256, the last being of strength equal to one-fourth that of the original methylene blue solution. The figures in the following table give the members of this series to which the various dialysates corresponded.

Membrane	1 day	2 days	3 days	4 days	5 days	6 days	7 days
70 (1)	0	0	0	0	0?	2—1	2
70 (2)	0	0	0	0	0	0?	trace
70 (3)	0	0	0	0	0	0?	trace
72·5 (1)	0	2—1	8—4	8	16	32—16	32–
72·5 (2)	0	2—1	4	8—4	8+	16–	16
72·5 (3)	0	2—1	4	8—4	8+	16–	16
75 (1)	trace	16–	32–	32+	64–	64	64+
75 (2)	trace	8	16+	32	64—32	64–	64
75 (3)	trace	8–	16	32–	64—32	64—32	64–
77·5 (1)	8–	32	64–	64	128—64	128–	128–
77·5 (2)	8—4	32	64–	64–	64+	128—64	128–
77·5 (3)	8	64—32	64	64+	128–	128–	128–
80 (1)	16+	64–	64+	64+	128–	128–	128–
80 (2)	16	64–	64+	64+	128–	128–	128–
80 (3)	32—16	64+	128—64	128	128	128	128

After 4–5 days no further dialysis takes place in the 80 % membranes, so that the less permeable 77·5 % membranes overtake them. On examining the liquid inside the 80 % dialysers on the 5th day, it was found that its

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strength was in each case about one-eighth that of the original solution. This loss in strength is due to the strong adsorption of dye by the membrane. The adsorptive capacity of these membranes increases with permeability. The amount of dye therefore which passes through the more permeable membranes in the above table is less than would be the case if the internal solutions were kept up to full strength.

A second test on a similarly large scale was carried out, 6 membranes of 75 %, 6 of 77·5 %, and 6 of 80 % being used. The results obtained were essentially the same as in the above case.

Throughout these experiments special attention was directed to the comparison of the most permeable membrane of a given alcohol number with the least permeable of the next higher number. On the basis of these observations, it can be stated that the most permeable membrane of a certain number was in all cases less permeable than the least permeable of those of number 2·5 % higher. Thus whatever be the variations among the individual membranes, these are not sufficient to mask the effects of the differential alcohol treatment—in other words in the region 70–80 % an alcohol treatment which varies by 2·5 % steps definitely produces continuous gradation in an average set of membranes. At the same time however the maximum variation among similarly treated members may be greater than the least gap which obtains between members of two lots the treatment of which differs by 2·5 %. Thus it is probable that with unselected membranes, 2·5 % more or less represents in the region 70–80 % the smallest variation of alcohol treatment which can be reckoned on to mask individual variations and to produce in all cases continuous gradation.

It is reasonable to ascribe the variations here recorded to variations in the original air-dried membranes (especially individual variations in thickness), and therefore it may be expected that with improvement in the method of manufacture a finer gradation will be rendered possible.

At higher points in the series a finer gradation in terms of alcohol percentage is possible. Thus from about 85 % upwards, continuous grading can be obtained by a series of alcohols varying by 1 %. In the region 90–96 %, especially towards the upper end, smaller differences in alcohol treatment will suffice to produce continuous grading. In the region 96–98 % imbibition increases so rapidly with alcohol percentage that a large range of membranes is included within this narrow limit. The accuracy of the grading in this region therefore would probably be found to be limited by the accuracy obtainable in preparing the alcohol mixtures.

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From 40–70 % a variation of 10 % in alcohol treatment produces continuous gradation. The air-dried membranes show differences in permeability to simple electrolytes, e.g. NaCl, some practically keeping back this salt, others allowing a very slow diffusion. These initial variations would be expected to influence more markedly the members of lower than of higher alcohol number. The accuracy attainable has not however been sufficiently tested in the case of these membranes of lower permeability.

By the use of this alcohol method, it is possible to prepare a series of membranes the members of which allow of slower and slower diffusion of a given substance. By continuing the series downwards a stage is reached where no diffusion at all of the particular substance takes place. Thus a given substance can be characterised by a number which represents the strength of alcohol required to produce a membrane which just prevents diffusion of the substance. Such a number may be termed the "alcohol index" for the particular substance. Some of these indices have been worked out, and are given in the following table:

Water	—	Safranine	75–77·5
NaCl, NH_4Cl		Dextrin	85–87·5
$KMnO_4$	30–40	Starch	90
Picric acid	35–40	Aniline blue	92
Potassium oxalate ...	60–70	Litmus (neut.)	93
Bismarck brown ...	65 (–)	Congo red	96
Methylene blue ...	70 (–)	Night blue	>96
Neutral red	72·5–75		

Water, sodium chloride and ammonium chloride all pass through air-dried membranes. Passage of water is shown by endosmosis, also by the fact that water evaporates from a sealed air-dried membrane when the latter is hung up in the air. The rate of passage is small, and individual variations are large in proportion. Some of these membranes only allow the faintest trace of sodium chloride to pass through in a week; in the case of others passage can be detected in two days. It is doubtful if any of these membranes can be prepared so as to allow passage to water, and at the same time to hold back completely simple chlorides.

The strong endosmotic effect is very troublesome in working with such substances as potassium oxalate, especially in molar concentration. The internal liquid tends to overflow and the levels have to be continually readjusted. Most of the crystalloids tested seem to possess a diffusibility similar to that of potassium oxalate. Such were $CuSO_4$, Na_2SO_4, $K_4Fe(CN)_6$. A 60 % membrane allowed a slow diffusion of glucose and of barium chloride. This part of the investigation was not however carried out at all thoroughly.

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It should be noted that membranes of index 50–60 % form very efficient semi-permeable membranes, i.e. while easily permeable to water, they hold back a large number of crystalloids. A membrane of this sort may be used to effect a dialytic separation of certain kinds of salts—thus sodium chloride can be readily dialysed away from a sodium chloride, sodium sulphate mixture. Whether such membranes would keep back these crystalloids under pressure remains to be seen.

It might be suggested that the relatively high impermeability of these membranes—say to molar sodium sulphate—is due to the effect of the strong salt solution upon the membrane, viz. to the withdrawal of water from the membrane by the osmotic action of the salt. This however is contradicted by the behaviour of strong sodium chloride solutions which should on this hypothesis have a similar dehydrating effect, by the magnitude of the endosmotic effect, and by the fact that a 60 % membrane after treatment with molar Na_2SO_4 is found to be still of number 60 % in its behaviour to potassium permanganate.

Only a very slow passage of starch is allowed by a 90 % membrane. Even a 92 % membrane to all practical purposes keeps back a starch solution during several days' dialysis. It is probable that many colloids are heterogeneous as regards degree of dispersion, so that a sharply defined "critical point" would not be expected.

The dialysates from 94 and 95 % membranes containing litmus show a peculiar feature. On comparing these with matching dilutions of the internal solution, they are found to give the same colour reaction to acids, but not to alkalis. Thus the addition of a drop of alkali to the matching solution produces a distinct blue; on similar treatment, the dialysates from 94 and 95 % membranes merely become slightly more violet. A similar behaviour, though not so marked, is seen in the dialysate from a 96 % membrane. It is suggested that litmus contains at least two fractions, one of which is more diffusible and gives a part only of the indicator reactions of the crude solution.

Equilibrium.

The question as to whether equilibrium between the collodion membrane and the alcohol solution has been reached in the 24 hours allowed for imbibition, has only been studied in the case of 72·5 % membranes. It was then found that no essential difference in permeability subsisted between membranes which had remained in contact with the alcohol solution for one day and membranes which had been similarly treated for 4 days. Thus in the case

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of 72·5 % membranes, a state of equilibrium or zone of slow change has been reached in 24 hours. Higher in the series it is probable that an imbibition equilibrium is reached in a shorter time, but here the slow change due to solution of the membrane becomes more marked. In the case of membranes lower than 70 %, it should not be taken for granted that equilibrium in imbibition is reached after 24 hours' immersion in the alcohol solution.

Uses of these membranes.

Some of the more obvious uses to which these membranes may be put are

(1) replacement of the copper ferrocyanide membranes in osmometric work;

(2) dialysis of intermediate colloids, e.g. dextrin: more generally, the examination and separation of digestion products;

(3) carrying out a more exact study than has yet been made of the relations between the phenomena of diffusion and adsorption.

An experiment of the type suggested in (2) will serve to illustrate the potentialities of the method.

Six 92 % membranes were made and set up as follows:

(1) and (2) 0·1 % starch solution + equal bulk of water,
(3) and (4) 0·5 % dextrin solution + equal bulk of water,
(5) and (6) 0·5 % dextrin solution + 0·1 % starch solution in equal bulks.

These were tested each day over a period of four days, when it was found that in no case could starch be detected in the dialysates, whereas dextrin of strength 0·05 % dialysed through in each 24 hours in the case of membranes (3), (4), (5) and (6).

On the fifth day, each dialyser was washed and set up alongside a 50 % and an 85 % membrane, with a mixture in equal parts of the following:

0·5 % starch solution,
0·5 % dextrin solution,
0·2 m glucose solution,
1 m sodium chloride solution.

After 24 hours' dialysis, the following scheme represents the condition of the dialysates:

Test	50 %	85 %	92 %	Internal
$AgNO_3$	fairly strong precipitate	strong precipitate	strong precipitate	strong precipitate
Fehling's solution	neg.	strong reduction	—	—
I_2 in KI	coloration = water control	coloration = water control	strong red coloration	intense blue

The application of these membranes to more difficult problems of analysis may be anticipated.

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All the work hitherto described was carried out with an 8 % collodion solution in equal parts of ether and alcohol. Other collodion solutions have been tested so far as to indicate what possibilities they present.

Acetone collodion; 8 % solution; dispersion takes place with rapidity, giving a solution which is more mobile than the corresponding ether-alcohol solution.

The membranes prepared from this solution in the ordinary way are white throughout, show normal amount of irregularity in thickness in different parts, have a small water percentage and comparatively low permeability in wet condition and show small shrinkage (6 %) on drying. In the wet condition the thickness is 8–10 μ, and after drying 5–7 μ.

When treated by the alcohol method, a more gradual variation with alcohol percentage is obtained, the interval 0 %—70 % in the case of ether-alcohol membranes corresponding with the interval 0 %—90 % with acetone membranes.

These membranes appear to be of practical utility, especially in respect of their more gradual grading below 90 %.

Ether collodion; 8 % solution; dispersion does not occur so rapidly as in acetone; the resultant solution is very viscous, more so than the ether-alcohol solution. The membranes were prepared in the usual way; they are white, give extremely small shrinkage on drying, and when freshly prepared are permeable only to water and a few salts. They are extremely difficult to prepare on account of the high impermeability which prevents the rapid access of water to the underlying layers. By the action of water the collodion film becomes hard and can with difficulty be removed from the tube.

On treatment with alcohol, these membranes show a behaviour similar to that of the acetone-collodion membranes described above, i.e. they respond more sluggishly to the alcohol treatment.

This last feature is considered to be of interest in respect of the general technique adopted. It would explain the tendency observed in certain membranes to be more permeable towards the closed end, where, on account of the conditions obtaining during their manufacture, they tend to contain a lower percentage of ether at the time of fixation in water.

Alcohol collodion. The 8 % solution was so viscous that a 4 % solution was employed. The latter is rather more fluid than the normal 8 % ether-alcohol solution.

The membranes were held for a few seconds in air, and then fixed in

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water. They are glass-clear and in the wet condition are highly permeable, e.g. to starch. In spite of the thin solution employed, wet membranes are extremely thick (200–300 μ), and have a very high water-content (dry weight $\doteq$ 100, wet weight = 1400). The shrinkage on drying is enormous (60–70 %).

The interest of these membranes is that they form the end term of the series; ether membrane → membrane of type (5, 0) → membrane of type (5, 1) → alcohol membrane.

Gradation by means of acetone-water mixtures.

No practicable results can be got by using acetone-water in place of alcohol-water mixtures. In 60 % acetone (and higher concentrations) membranes of any type become unmanageably soft (the clear normal type of membrane becoming white in this process); in 40 % acetone, the membranes soften, shrink to very small dimensions, and on transferring to water become hard and highly impermeable.

Membranes made inside the tube.

These can be prepared conveniently as follows. Two tubes of the same diameter are passed through a cork as in Fig. 1. One of these is filled with 8 % collodion solution. On turning upside down and allowing to drain, a film is left inside the upper tube. Fifteen minutes drainage was allowed. The tube with the film is then taken out, and air blown in by means of a pipette until the whole film is nearly dry (one minute is about sufficient); the free end of the membrane is now disengaged with a knife and a little further blowing causes the whole membrane to come away. These membranes are simply dried out in air without coming in contact with water. They are of the clear alcohol type, but do not show shrinkage as they had already almost dried while in contact with the glass, which restrains shrinkage in a mechanical way.

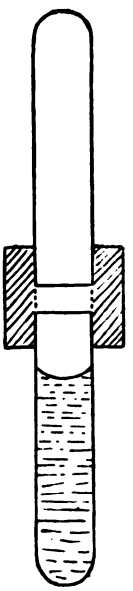

Fig. 1

These membranes can with skill be prepared of great uniformity (apart from the usual tendency to be thinner at the closed than at the open end), they are very thin, they can be differentiated in alcohol in the usual way, and their use is recommended in cases where a rapid dialysis is imperative and where adsorption of a particular substance on the membrane is required to be

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reduced as much as possible. These membranes, on account of their extreme thinness, are rather fragile.

Thin sheet collodion can be readily prepared in this way, viz. by forming a membrane inside a large cylindrical vessel and treating in the above manner. After drying, the film is simply cut down one side with a knife and taken out.

Method of grading membranes by controlling the amount of drying.

This method was the one first adopted. It bears certain resemblances to Schoep's method. In fact, in the early stages, glycerol solutions were used, the wet membranes as prepared in the first instance being soaked in glycerol solutions of different strengths and then hung up in air to dry. The higher the percentage of glycerol the greater the permeability of the resultant membrane.

This method was later superseded by the following. A solution of $CaCl_2$, as nearly as possible saturated and with alkalinity neutralised, was prepared, filtered, and placed in the bottom of a large desiccator. This solution may be designated *A*. The wet membranes were soaked for 24 hours in various dilutions of *A*, and then hung up over the concentrated solution in the desiccator; the latter is then exhausted. By this means the attainment of equilibrium is expedited. It is obvious that a membrane which has imbibed a 5 % dilution of *A* loses more water in reaching equilibrium with *A* than a membrane which has been soaked in a 10 % dilution, and would accordingly be expected to be less permeable. The membranes are finally washed and are then ready for use.

The practical features of the process are as follows:

The $CaCl_2$ solution should be as strong as possible: with weak solutions the attainment of equilibrium is slower. Before the membranes are hung up to dry in the desiccator, all excess drops of the diluted $CaCl_2$ solution must be wiped off. Only in this way can uniformity be obtained.

As regards results, this method is distinctly good. It is necessary however that the membranes before treatment be of uniform permeability throughout. Any marked variations will be reproduced in the graded membranes. In fact in the present work the variations in different parts of the freshly prepared membranes as described on p. 596 were first discovered by the variations which appeared in different parts of the membranes, the permeability of which had been reduced in this way. In order to secure uniform membranes to begin with, the freshly prepared membranes were soaked in a 94 % alcohol solution.

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A membrane which has been soaked in 94 % alcohol, then graded down by using a 7·5 % *A* solution just holds back methylene blue, i.e. it corresponds to a membrane treated with 70 % alcohol.

Apart from its laboriousness and its limited applicability, this method may be objected to inasmuch as the removal of the calcium chloride from the graded membrane may be slow, and may not take place at all in some cases. This objection could be met by using strong solutions of sodium chloride instead. There is a possibility that such a method may be of use in preparing membranes for osmometric work.

Summary.

Collodion thimbles of regularly increasing degree of permeability may be prepared by soaking air-dried thimbles in alcohol-water mixtures of increasing alcohol content.

The diffusive capacity of any substance through collodion may be specified in terms of the alcohol strength required to produce the membrane which just prevents its passage. This may be termed the "alcohol index" of the substance.

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